

***Aphanizomenon flos-aquae* Protects against the Biochemical Changes Induced in Rats Consuming a Mixture of Food Additives**

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Abstract

Various additives are added to food products to attain the desired properties such as flavor, preservation, and sweetening. The goal of this research was to investigate the biochemical alterations in the rats consuming a food additives mixture (FAM) containing sodium nitrate, fast green, and glycine; and the potential protective impact of *Aphanizomenon flos-aquae* (*A. flos-aquae*). Thirty male albino rats were classified into three equal groups (n=10) Group I: the control, group II: FAM; group III: FAM + *A. flos-aquae*. After 30 days, the biochemical analysis revealed a significant increase in the liver (ALT and AST) and kidney (creatinine and urea) toxicity markers. The FAM significantly increased total cholesterol, triglycerides, and low-density lipoprotein. The mixture significantly decreased body weight, high-density lipoprotein, total protein, albumin, globulin, and testosterone. The FAM had no impact on serum glucose, insulin, and thyroid hormones (T3 and T4). *A. flos-aquae* significantly protected against these biochemical alterations. The current findings showed that *A. flos-aquae* can protect against the severe impacts of a FAM on key biochemical markers.

Keywords: *Aphanizomenon flos-aquae*, Food additives, Biochemical changes, FAM

Introduction

Currently, the use of food additives have been widely increased for numerous reasons including improving products sensory characteristics and palatability enhancers as flavor, taste, color, texture, and smell; increasing the shelf life and preventing products spoilage; making food products easy to prepare and cook;

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achieving the consistency needed for large-scale production; and increasing consumer attractiveness (Bawazir, 2016; Alsudani & Alhamadawi, 2020). Although the several advantages of using food additives, however consuming food additives-containing products for a long time may induce several health problems as growth retardation; allergies; indigestion; anemia; neurotoxicity; disorders in renal, liver, and spleen; as well as cancerous diseases (Elbanna *et al.*, 2017; Amin & Al-Shehri, 2018). It may also cause intestinal irritation, immune system disorders; hypothalamic-pituitary-testis axis inhibition that leads to reproductive disorders; and oxidative stress (Soltan & Shehata, 2012; Alsudani & Alhamadawi, 2020).

Sodium nitrate is a food preservative used as color fixatives, especially in processed meat, fish, and poultry products (Cockburn *et al.*, 2014). It has antimicrobial properties to inhibit the growth of microbes that release neurotoxin-producing food toxicity and threaten human health (Marianski *et al.*, 2009; Ziarati & Arbabi-Bidgoli, 2014). However, after consuming foods that contain sodium nitrate, some of the nitrates are converted to nitrite, which combines with amines and amides in the acidic environment of the stomach to form nitroso compounds that increase intracellular reactive oxygen species (ROS) (Erkekoglu & Baydar, 2010). Elevated ROS may cause DNA, cell membrane, and proteins damage and may induce several diseases as hepato-renal toxicity (Sharma *et al.*, 2012; Baek *et al.*, 2015).

Fast green is a synthetic organic food dye used as a coloring agent in numerous food products as candies, beverages, ice cream, dairy products, and baked goods. The consumption of such products may have hepatic-nephron carcinogenesis and tumorigenic effects via the generation of hydroperoxide isomers. In addition, it may irritate skin, eyes, respiratory tract, and digestive tract (Ashour & Abdelaziz 2009; Helal *et al.*, 2017). As well as may induce hyperactivity disorder and attention deficit in children (Lau *et al.*, 2006; Tripathi *et al.*, 2007).

Glycine is a sweet-tasting crystalline used as a flavoring and preservative food additive that may owe to its complexation to metal ions (Meléndez-Hevia *et al.*, 2009). It induced neurogenic function changes in glycine-treated animals that disrupted the hypothalamic-pituitary-testis regulatory axis (Helal *et al.*, 2000).

Natural antioxidant ingestion has been proved to improve the human's efficiency in stressful conditions (Samarghandian *et al.*,



2013). Blue-green algae, especially *Aphanizomenon flos-aquae* (*A. flos-aquae*) and *Spirulina sp.* have been used as source materials for drug development, and they are commercially consumed as dietary supplements (Schaap *et al.*, 2012). *A. flos-aquae* is a nutrient-dense food, containing abundant amounts of vitamins, minerals, chlorophyll, carotenoids, and phycocyanin. Phycocyanin possesses powerful antioxidant and anti-inflammatory activities (Khuantrairong & Traichaiyaporn, 2012; Scoglio *et al.*, 2014; Li *et al.*, 2016).

In addition, *A. flos-aquae* has potent probiotic compounds that enhance human health (Wu *et al.*, 2012). Several studies proved the antioxidant, radioprotective, hypolipidemic, and anti-inflammatory activities of *A. flos-aquae* (Yang *et al.*, 2011; Venkatesan *et al.*, 2012; Eid *et al.*, 2016).

Therefore, this study aims to evaluate the biochemical alterations in the rat's ingestion of a food additives mixture (FAM) (sodium nitrate, fast green, and glycine) and the potential preservative impact of *A. flos-aquae*.

Materials and Methods

Chemicals

Aphanizomenon flos-aquae (*A. flos-aquae*-Klamath 350 mg/ each capsule, STEM Technology Health Sciences, San Clemente, CA, USA) were obtained from the German Egyptian Pharmaceutical Company. Food additives (fast green, glycine, and sodium nitrate) were obtained from Sigma-Aldrich Co, USA. Enzymatic colorimetric kits were provided from Centronic Chemicals Co, Germany. ELISA kits were obtained from Glory Science Co. (Ltd. Del Rio-TX-USA).

Animals

Thirty male albino rats weighing 120-140 g were obtained from the Animal Unit, Al-Azhar University, Cairo, Egypt.

Experimental Design

Animals were housed in stainless steel cages under hygienic conditions. They were fed standard chew and drinking water *ad libitum*. After the adaptation week, the rats were classified into 3 equal groups (n=10/each). 1- Control; rats were orally ingested phosphate-buffered saline. 2- FAM; rats were ingested with a FAM (10 mg/kg sodium nitrate, 12.5 mg/kg fast green, and 12.5 mg/kg glycine). 3- FAM + *A. flos-aquae*; rats were ingested with the previous FAM plus *A. flos-aquae* (94.5 mg/kg) (Abu-Amara *et al.*, 2016).

Over the experimental periods (30 days), the body weights were recorded. Blood samples were taken from each rat after anesthesia at the end of the experiment, then centrifuged at 5000 rpm for 10 minutes. Sera were separated and stored at -80 °C until utilized for the biochemical analysis.

Quantification of Glucose, Insulin, and HOMA-IR

Serum fasting glucose concentration was quantified using an enzymatic colorimetric kit. Insulin level was quantified using an ELISA kit. HOMA-IR was calculated as follows (Mohamed *et al.*, 2021):

$$\text{HOMA-IR} = \frac{\text{Fasting glucose (mg/dl)} \times \text{Insulin } (\mu\text{u/L})}{405} \quad (1)$$

Quantification of Protein Metabolism

Serum total proteins (TP) and albumin concentrations were quantified using enzymatic colorimetric kits following the manufacturer's directions. Globulin concentration and albumin/globulin ratio were calculated according to the formula (Mohamed *et al.*, 2021).

Quantification of Liver and Renal Functions

Liver function enzymes (aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities) and renal functions parameters (creatinine and urea levels) were quantified using enzymatic colorimetric kits following the manufacturer's directions.

Quantification of Lipid Profile

Serum lipid profiles (total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) were quantified using enzymatic colorimetric kits. However, TC/HDL and LDL/HDL ratios were calculated using formulas (Friedewald *et al.*, 1972).

Quantification of Testosterone Level

Serum testosterone level was quantified using an ELISA kit according to the manufacturer's directions.

Quantification of Thyroid Hormone Levels

Serum T3 and T4 were quantified using ELISA kits according to the manufacturer's instructions.

Statistical Analysis

Results were introduced as mean \pm SE. Analysis was conducted using the SPSS program, version 25. The Bonferroni test was used to compare between groups (p-value of ≤ 0.05 was considered significant).

Results and Discussion

Effect of *Aphanizomenon flos-aquae* (*A. flos-aquae*) on Body Weight Change, Glucose, Insulin, and HOMA-IR

In the FAM group, the body weight was significantly lower than that of the control group, as the change in body weight decreased significantly (22.47%) relative to the control group. But in rats

consumed the FAM + *A. flos-aquae* combination, body weight returned to the control group level (**Figure 1a**).

Rats consumed the FAM did not impact glucose, insulin, and HOMA-IR levels relative to the control group. Similarly, rats that consumed the FAM + *A. flos-aquae* combination did not impact insulin and HOMA-IR levels relative to the FAM group. But in rats treated with the FAM + *A. flos-aquae*, glucose level increased relative to the FAM group (**Figures 1b, c, and d**).

Effect of A. flos-aquae on Total Protein, Albumin, Globulin, and Albumin/Globulin Ratio

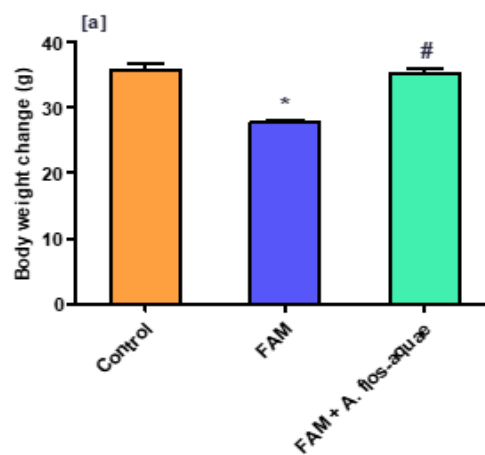
In the FAM group, total protein, albumin, and globulin were significantly lower than that of the control group, as all were decreased significantly (27.99%, 20.63%, and 39.05%, respectively) relative to the control group. Rats consumed the FAM + *A. flos-aquae* combination significantly increased total protein, albumin, and globulin levels relative to the FAM group. But in rats that consumed the FAM + *A. flos-aquae* combination, all parameters were returned to the control group level (**Figures 2a, b, and c**). The albumin to globulin ratio was not altered in any groups (**Figure 2d**).

Effect of A. flos-aquae on Liver Function (ALT and AST)

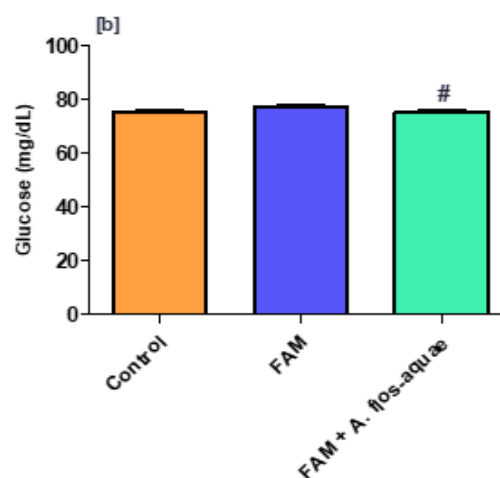
In the FAM group, ALT and AST levels were significantly increased than that of the control group, as both were increased significantly (86.94% and 25.7%) relative to the control group. Rats consumed the FAM + *A. flos-aquae* combination significantly decreased ALT and AST levels relative to the FAM group. But in rats consumed the FAM + *A. flos-aquae* combination, both enzymes were returned to the control group level (**Figures 3a and b**).

Effect of A. flos-aquae on Serum Lipids Profile

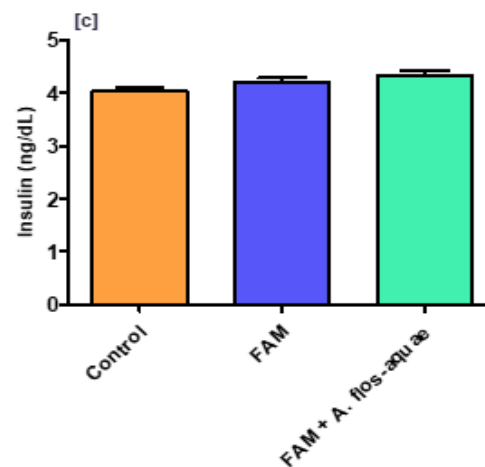
In the FAM group, TG, LDL, VLDL, LDL/LDH, and TC/HDL levels were significantly increased than that of the control group, as both were increased significantly (3.9%, 27.83%, 3.23%, 37.50%, and 13.18%, respectively) relative to the control group. In the FAM group, HDL level was significantly decreased than that of the control group, as it was significantly reduced (7.65%) relative to the control group. Rats consumed the FAM + *A. flos-aquae* combination significantly decreased TG, VLDL, LDL/LDH, and TC/HDL levels relative to the FAM group. All lipids in rats that consumed the FAM + *A. flos-aquae* combination were returned to the control group level except LDL level which was still higher than the control and FAM groups' levels (**Table 1**).



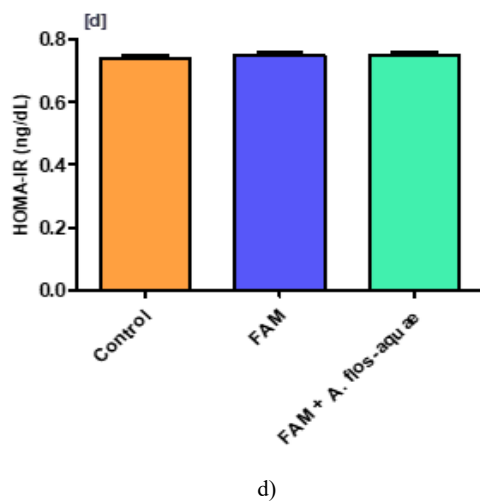
a)



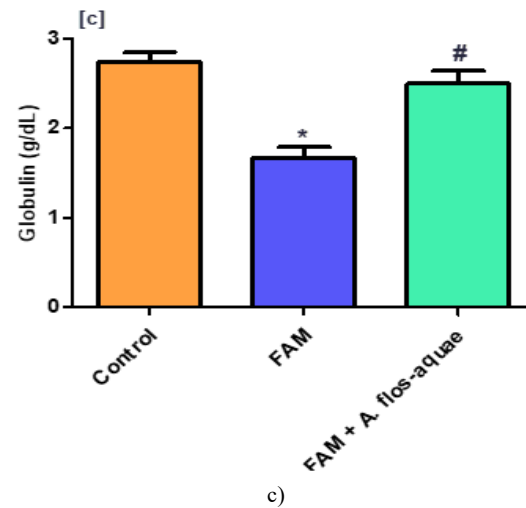
b)



c)

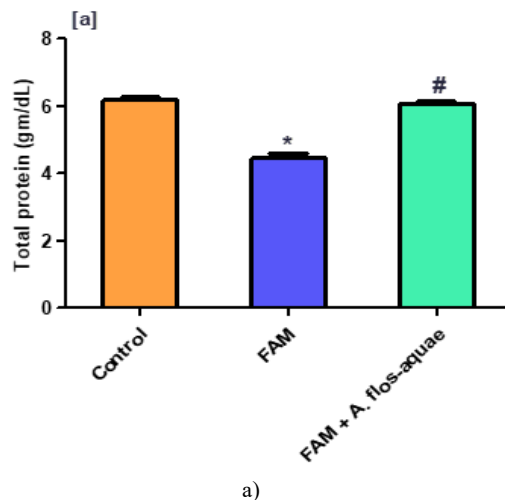


d)

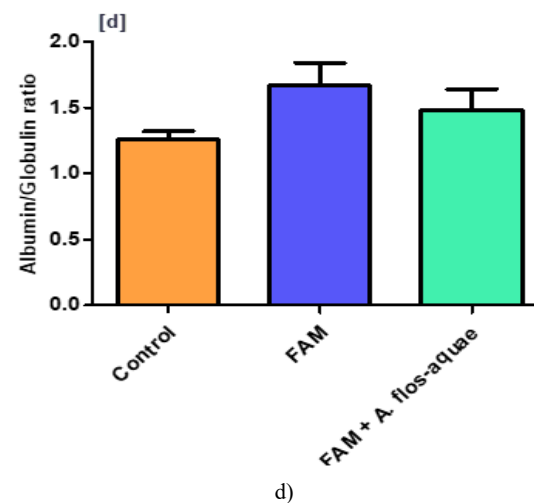


c)

Figure 1. Effect of *A. flos-aquae* on [a] body weight change, [b] glucose, [c] insulin, and [d] HOMA-IR quantified in food additives mixture (FAM) (fast green, glycine, and sodium nitrate) treated rats. Bars represent mean \pm standard error. *represents significant relative to control rats, # represents significant relative to FAM rats. $p \leq 0.05$.

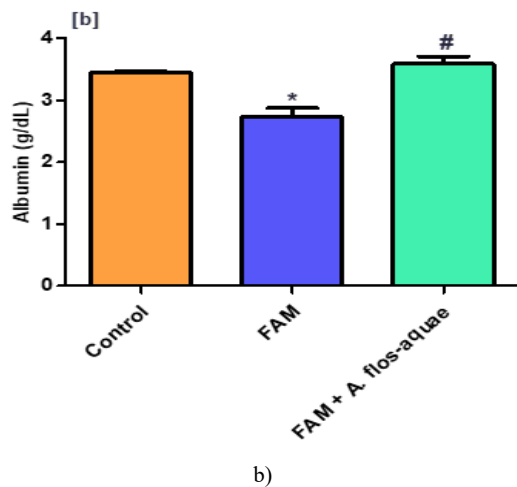


a)

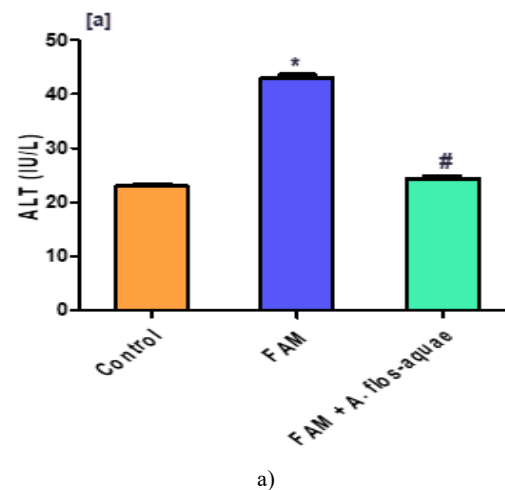


d)

Figure 2. Effect of *A. flos-aquae* on [a] total protein, [b] albumin, [c] globulin, and [d] albumin/globulin ratio quantified in food additives mixture (FAM) (fast green, glycine, and sodium nitrate) treated rats. Bars represent mean \pm standard error. *represents significant relative to control rats, # represents significant relative to FAM rats. $p \leq 0.05$.



b)



a)

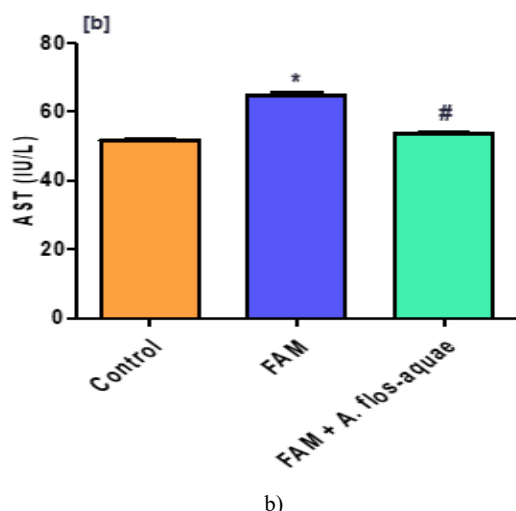


Figure 3. Effect of *A. flos-aquae* on liver function [a] ALT and [b] AST quantified in food additives mixture (FAM) (fast green, glycine, and sodium nitrate) treated rats. Bars represent mean \pm standard error. *represents significant relative to control rats, #represents significant relative to FAM rats. $p \leq 0.05$.

Effect of *A. flos-aquae* on Kidney Function (Creatinine and Urea)

In the FAM group, creatinine and urea levels were significantly increased than that of the control group, as both were increased significantly (75.82% and 38.62%) relative to the control group. Rats consumed the FAM + *A. flos-aquae* combination significantly decreased creatinine and urea levels relative to the FAM group. But in rats consumed the FAM + *A. flos-aquae* combination, both were returned to the control group level (Figures 4a and b).

Table 1. Effect of *A. flos-aquae* on serum TC, TG, HDL, LDL, VLDL, LDL/HDL ratio, and TC/HDL ratio quantified in food additives mixture (FAM) (fast green, glycine, and sodium nitrate) treated rats.

Group	Control	FAM	FAM + <i>A. flos-aquae</i>
TC (mg/dl)	80.00 \pm 1.14	83.00 \pm 0.70	82.00 \pm 0.70
TG (mg/dl)	75.78 \pm 0.36	78.20 \pm 0.58*	76.40 \pm 0.53#
HDL (mg/dl)	43.75 \pm 0.53	40.40 \pm 0.50*	42.29 \pm 0.48#
LDL (mg/dl)	21.09 \pm 0.78	26.96 \pm 0.85*	24.43 \pm 1.10
VLDL (mg/dl)	15.15 \pm 0.07	15.64 \pm 0.11*	15.27 \pm 0.10#
LDL/HDL	0.48 \pm 0.01	0.66 \pm 0.02*	0.57 \pm 0.03#
TC/HDL	1.82 \pm 0.01	2.06 \pm 0.03*	1.93 \pm 0.03#

Data represent mean \pm standard error. *represents significant relative to control rats, #represents significant relative to FAM rats. $p \leq 0.05$.

TC: total cholesterol; TG: triglyceride; HDL: high-density lipoprotein; LDL: low-density lipoprotein

Effect of *A. flos-aquae* on Serum Testosterone Level

In the FAM group, the testosterone level was significantly decreased than that of the control group, as it was significantly reduced (27.57%) relative to the control group. Rats that consumed

the FAM + *A. flos-aquae* combination significantly increased testosterone level relative to the FAM group. But in rats that consumed the FAM + *A. flos-aquae* combination, the testosterone level was still decreased than the control group level (Figure 5).

Effect of *A. flos-aquae* on Serum Thyroid Function (T3 and T4)

Rats that consumed the FAM did not impact T3 and T4 levels relative to the control group. Similarly, rats that consumed the FAM + *A. flos-aquae* combination did not impact T3 and T4 levels relative to the FAM group (Table 2).

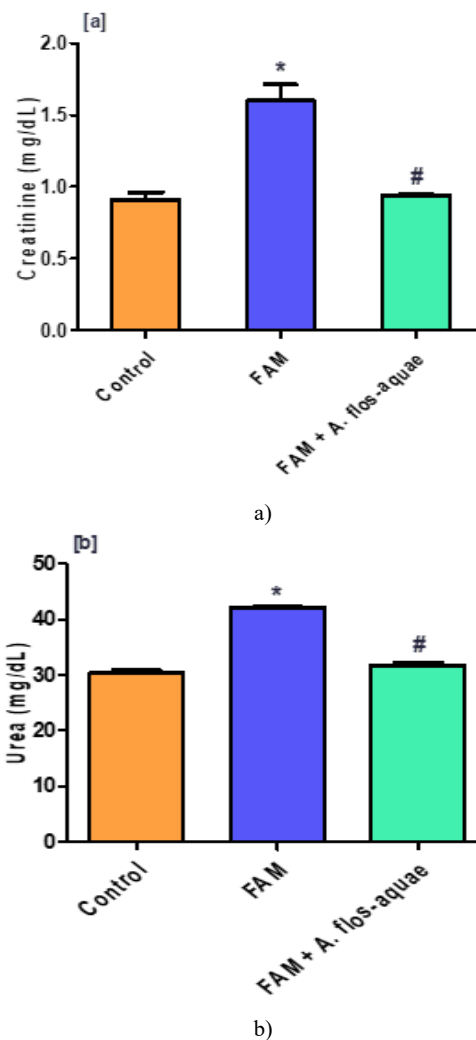


Figure 4. Effect of *A. flos-aquae* on kidney function [a] creatinine and [b] urea quantified in food additives mixture (FAM) (fast green, glycine, and sodium nitrate) treated rats. Bars represent mean \pm standard error. *represents significant relative to control rats, #represents significant relative to FAM rats. $p \leq 0.05$.

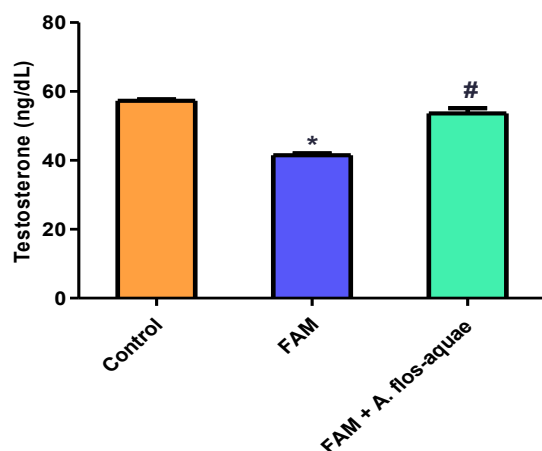


Figure 5. Effect of *A. flos-aquae* on serum testosterone quantified in food additives mixture (FAM) (fast green, glycine, and sodium nitrate) treated rats. Bars represent mean \pm standard error. *represents significant relative to control rats, #represents significant relative to FAM rats. $p \leq 0.05$.

Table 2. Effect of *A. flos-aquae* on serum T3 and T4 quantified in food additives mixture (FAM) (fast green, glycine, and sodium nitrate) treated rats.

Group	T3 (ng/dl)	T4 (ng/dl)
Control	108.22 \pm 0.83	4.57 \pm 0.15
FAM	109.74 \pm 0.40	4.29 \pm 0.12
FAM + <i>A. flos-aquae</i>	111.64 \pm 1.89	4.21 \pm 0.10

Data represent mean \pm standard error.

The study of the impact of various FAM consumed by humans on the blood components of experimental animals as variations from normal values has remained a viable tool for investigating the impact of these elements on human health. The extensive usage of various food additives has resulted in harmful health impacts that must be monitored in the future (Tawfek *et al.*, 2015; Helal *et al.*, 2019). The current results showed a significant reduction in body weight in the rats that consumed the FAM (fast green, glycine, and sodium nitrate). According to Aboel-Zahab *et al.* (1997) fast green is a synthetic food colouring that leads to significant weight loss in rats. Nitrate can also contribute to weight loss by reducing food intake. As it affects the neurological control of eating behavior causing a decrease in hepatic growth hormone receptors. Consequently, a deficiency of plasma somatomedins occurs which results in a decrease in body growth (Helal *et al.*, 2000; Akasha *et al.*, 2015; Mohamed *et al.*, 2021).

The results of the study showed that rats consuming the FAM did not affect serum glucose, insulin, or HOMA-IR levels. Treatment of FAM rats with *A. flos-aquae* significantly reduced serum glucose level compared to the FAM rats. Inconsistent with the current findings, Abdelhafez *et al.* (2018) showed that the improvement in diabetic rats treated with *A. flos-aquae* extract could be attributable to beta cell stimulation.

The findings revealed significant reductions in total protein, albumin, and globulin levels in the rats who consumed the FAM.

Previous research indicated that the fast green decreased total protein by increasing amino acid deamination (Amin *et al.*, 2010). The harmful effects of sodium nitrate may be due to the generation of nitric oxide or peroxynitrite, which oxidizes proteins and lipoproteins; thereby reducing protein concentrations. Nitrate has been shown to lower total serum protein primarily through its effects on the liver causing necrotic changes and suppression of the oxidative phosphorylation pathway. That initially reduces the energy intake available for the production of proteins and other biochemical activities of proteins (El-Wakf *et al.*, 2011). The current study found that combining *A. flos-aquae* with FAM considerably boosted total protein, albumin, and globulin levels. This could be because some *A. flos-aquae* components have antioxidant properties (Elmalawany *et al.*, 2014).

The present results indicated a marked rise in serum ALT and AST levels in response to FAM administration. In agreement with these results, Amin *et al.* (2010) stated doses of artificial colours such as fast green that were too low or too high led to a significant increase in serum ALT and AST. They attributed these changes to hepatocellular damage caused by the toxic properties of these artificial dyes. In rats given large doses of food dye, the activity of liver serum enzymes (AST and ALT) elevated, indicating increased permeability, damage, and degradation of liver cells. Azo dyes (fast green) have the potential to harm liver cells and mitochondrial membranes (Mohamed *et al.*, 2016). Previous research found that sodium nitrate-treated rats had higher AST and ALT levels due to the development of a peroxynitrite free radical, which caused liver damage and was involved in liver cell death (Husain *et al.*, 2006).

Rats that consumed FAM showed noticeable alterations in their serum lipids levels, with marked increases in TC, TG, LDL, VLDL, TC/HDL, and LDL/HDL and a drop in HDL. Nitrate has been found to demonstrate a direct effect on the liver, which plays a key role in cholesterol metabolism and elevated cholesterol levels (Husain *et al.*, 2006). Higher levels of Acetyl CoA and the migration of free fatty acids from adipose tissue into the circulation may be linked to increased cholesterol synthesis (Abu-Aita & Mohammed, 2014). The probable clarification for these observed elevations in most lipid profiles could be the direct or indirect effects of the FAM on lipid metabolism or lipid peroxidation. The high concentrations of TC may indicate an alteration in the structure and function of the membrane that affects its fluidity, permeability, and the activity of associated enzymes, as well as the transport system (Hassan & Yousef, 2010). The current study found that combining *A. flos-aquae* with FAM lowered all lipid parameters, and increased HDL, which was increased. Blue-green algae have been shown to inhibit intestinal cholesterol absorption, decrease hepatic lipids, and attenuate plasma TC and TG concentrations (Kushak *et al.*, 2000).

The present results exhibited renal dysfunction in response to the administration of the FAM, which was evidenced by a significant increase in serum levels of urea and creatinine. Consistent with these results, serum urea, creatinine, and uric acid concentrations showed a marked increase in rats treated with a mixture of monosodium glutamate and aspartame (El-Ezaby *et al.*, 2018).

Increased protein catabolism and amino acid deamination by fast green could be a plausible cause for higher urea levels (Helal *et al.*, 2000). The increase in urea and creatinine levels could be attributed to oxidative stress generated by sodium nitrate therapy (Al-Gayyar *et al.*, 2016; Ansari *et al.*, 2018). Treatment of the rats group with food mixtures containing *A. flos-aquae* markedly ameliorated serum creatinine and urea concentrations. This could be due to its antioxidant activities, which enhance kidney function by mitigating the decrease in glomerular filtration and renal hemodynamics mediated by oxidative stress (Kuriakose & Kurup, 2008).

Moreover, the findings of the current study showed that daily consumption of the FAM did not affect thyroid hormones levels (T3 and T4). This effect could be attributed to the antagonistic impact of sodium nitrate, that decreases T3 and T4, and fast green, which raises them (Sun *et al.*, 1991).

A significantly lower testosterone level was found in the food additives group. Previous research of food additives was shown to reduce androgens by influencing the hypothalamic-pituitary-testicular axis (Bodnár *et al.*, 2001). The central nervous system of glycine-treated rats showed neurogenic functional changes in the hypothalamus, resulting in lower LH, FSH, and testosterone (Singh *et al.*, 1995). In the current study, *A. flos-aquae* treatment resulted in a significant improvement in testosterone levels compared to the mixed food additives group. These results were similar to those of Eid *et al.* (2016) that showed an improvement in testosterone levels with *A. flos-aquae* treatment in rats exposed to radiation, attributed to a decrease in lipid peroxidation.

Conclusion

The current results revealed that treatment with a mixture of food additives in rats caused a destructive effect on body weight, liver, and kidney functions. The level of harmful serum lipids was also increased, while good cholesterol and testosterone levels were decreased. The food additives mixture exhibited no effect on serum levels of glucose, insulin, and thyroid hormones. On the other hand, the treatment with *A. flos-aquae* resulted in a significant improvement in all of the measured parameters.

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Conflict of interest: None

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Ethics statement: The experimental design was accepted by the Care and Use Committee of the Faculty of Science, Al-Azhar University, Cairo, Egypt.

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