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Anti-obesity Effects of Foods Producing Hydrogen Sulfide in Rats Fed a High-Fructose Diet

Azza Ahmed[1,2*], Jihan Hasanen[1], Azza Abdalla[1], Reham Alattar[3], Tarek Khamis[4], Khalifa El-Dawy[2]

[1]Chemistry Department, Faculty of Science, Suez Canal University, Ismailia, Egypt, 41522.

[2]Biochemistry Department, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt, 44511.

[3]Fellow in the Health affair, Student's Hospital, Zagazig University, Zagazig, Egypt, 44511.

[4]Pharmacology Department, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt, 44511.

***Correspondence**
Corresponding author: Azza Ahmed
E-mail address: azza.orabi85@gmail.com

Abstract

The goal of this research was to determine the impact of garlic oil and leek powder on serum lipid levels and on sonic hedgehog (*SHH*) signalling pathway in a rat model of hyperlipidemia, to offer evidence supporting the use of diet as a means of preventing hyperlipidemia, and to further investigate the possible health advantages of these two H2S-donors. In this study, 80 males Wistar rats were divided into eight groups, for a period of 18 weeks, the rats were orally dosed with garlic oil and leek powder. Obesity indicators, serum lipid levels, some biochemical, molecular parameters and histopathology of liver tissues were determined. Compared to rats in the obese model groups, the garlic oil and leek powder reduced cholesterol, triacylglycerol, low-density lipoprotein-cholesterol (LDL-c) and very low density lipoprotein-cholesterol (VLDL-c) levels in the serum ($P < 0.05$). The garlic oil and leek powder also helped reduce hepatic steatosis and enhance lipid profiles. A reduction was found in the gene expression in the hepatic homogenate of Patched 1 (*Ptch1*), *SMO*othened (*SMO*), glioma-associated oncogene homolog 1 (*GLI1*), Sonic hedgehog protein (*SHH*), Toll-like receptor 4 (*TLR 4*), and nuclear factor kappa kB (*NF-kB*). On the other hand, there was a significant upregulation in the mRNA expression of hedgehog-interacting protein (Hhip-1), and *miRNA 26a* which is also a dose- and time-dependent manner. The obtained findings suggested that both garlic oil and leek powder as H_2S donor had anti-obesity capabilities that can mitigate the effects of an HFD.

KEYWORDS
Hydrogen sulphide, Obesity, Gene expression, Sonic hedgehog signaling pathway, *miRNA 26a*.

INTRODUCTION

Historically, hydrogen sulphide (H_2S) has been considered a poisonous gas for living organisms (Kimura, 2013). Adipocytes and fat tissues produce H_2S endogenously, and this gas promotes adipose tissue development. It is formed inside the body from L-cysteine and in the gut microbiome by a number of sulfate-reducing bacteria. These days, it's understood to be a gaseous mediator that affects the progression of cardiovascular and metabolic illnesses (Khattak *et al*., 2021; Wang *et al*., 2021; Peleli *et al*., 2022). The role of H_2S in the pathogenesis of obesity, type 2 diabetes, and cardiovascular illnesses is becoming increasingly well supported. An important area of study is the regulation and control mechanisms of H_2S homeostasis. Garlic (*Allium sativum*), and leek (*Allium ampeloprasum*) are examples of the allium vegetable family commonly consumed by humans. The breakdown of S-alkenyl-L- cysteine sulfoxides is the primary source of sulphur compounds in these plants that act as sources of H_2S (Rose *et al*., 2021; Ahmed *et al*., 2023).

Obesity is a disease occurred due to an improper ratio between the energy ingested in food and energy expenditure, with excessive fat storage and a body mass index (BMI) over 30 kg/m^2. Fat cells expand or multiply to accommodate the surplus energy. The pathologic lesion of obesity is the proliferation and enlargement of fat cells. Excessive adipose tissue buildup is a well-known contributor to metabolic impairment, and it has been associated to serious medical issues (Mezouari *et al*., 2020).

MicroRNAs (miRNAs) are short stretches of noncoding RNA that have a negative role in post-transcriptional gene regulation. Many microRNAs, including miRNA-27b, and miRNA-155, have been linked to metabolic diseases like obesity and diabetes (Murri *et al*., 2013; Elnagar and El-Dawy, 2019). The *SHH* signalling system plays a role in coordinating several processes essential for normal embryonic development. Adipocyte differentiation, cancer, diabetes, and obesity are only some of the physiological and pathological processes that have been linked to *SHH* pathway disruption. Pharmacological regulation of the *SHH* system has been shown to have the potential to enhance patient outcomes in metabolic diseases (Elnagar *et al*., 2018; Garg *et al*., 2022).

Toll-like receptors (TLRs), are thought to activate numerous inflammatory signalling pathways and help in pathogen identification and induce immunity. TLR4 share in the development of obesity-related inflammation in recent research. Obesity and the metabolic syndrome have prompted substantial research into the role of the nuclear factor kappa kB (*NF-kB*) signalling (Ye *et al*., 2022).

The current lack of knowledge about H_2S's part in obesity is a major problem. Consequently, the present study focused on the effect of H_2S on lipolysis and insulin resistance in obese rat induced by high fructose diet (HDF).

MATERIALS AND METHODS

Preparation of garlic oil and leek powder

Garlic oil from fresh *Allium sativum* bulbs, was distilled with oil extractor and an electric heater thermostat for 2 hours on finely chopped fresh garlic cloves that had been steeped in distilled water at 35°C for 4 hours. Anhydrous sodium sulphate was used to remove water from the extracted essential oils, and the resulting oils were then stored in a cool, dark place until needed (Yang *et al*., 2018).

Egyptian leek (Allium porrum L.), was procured at a local market in Egypt. The acquired leek was in pristine condition, so it was cut into chips and dried in a 40°C oven for a full week. Commercial blenders were used to turn the dried chips into a powder. The powdered sample was placed in a sealed container and frozen for later analysis (El-Khabery *et al*., 2016).

Animals and study protocol

The Experimental animal house of the Faculty of Veterinary Medicine, Zagazig University provided 80 adult males Wistar rats weighing 150-170 g. The experimental protocol was approved by IACUC Committee with approval number (REC 51/2022).

Obesity was induced by feeding animals with HDF for a period of 10 weeks. The HDF is made by adding 60% wt/wt fructose to a conventional rat diet (Maithilikarpagaselvi *et al*., 2016), and 60% of the diet's calories come from fructose. Fructose-induced obesity was evaluated by an increase in fasting blood glucose, insulin, and triglycerides 10 weeks after a high-fat meal was administered.

Group I (Control): they were fed a regular diet of food during the study. Group II (Obese) consumed a HDF. Group III (THGO): obese rats were given a high-dose of garlic oil (92.6 mg/kg·bw/d); the garlic oil was dissolved in 1% Tween 80 and administered to the rats every day by intragastric administration for 8 weeks (Yang *et al*., 2018). Group IV (TLGO): obese rats were given a low-dose of garlic oil (11.6 mg/kg·bw/d); every day by intragastric administration for 8 weeks (Yang *et al*., 2018). In Group V (Tleek), the obese rats were given diet containing 11% Egyptian leek powder for 8 weeks (El-Khabery *et al*., 2016). In group VI (PHGO) rats were fed on HDF and high-dose of garlic oil (92.6 mg/kg·bw/d) for 10 weeks as prophylactic dose. In group VII (PLGO) rats were fed on HDF and low-dose of garlic oil (11.6 mg/kg·bw/d) for 10 weeks as prophylactic dose. In group VIII (PTleek) rats were fed on HDF and diet containing 11% Egyptian leek powder for 10 weeks as prophylactic dose.

Measuring body weight and BMI

Body weight was measured at day zero, after 10 weeks, and at 18-week (end of the study). BMI was calculated by dividing body weight (g) on length (cm^2).

Collection of blood and tissue samples

At the end of the experiment and after 12 hours of fasting, all rats in the experiment were anaesthetized with pentobarbital sodium. Blood samples were collected from orbital venous plexus. Two types of blood samples were collected, the first, whole blood sample that was collected on EDTA and used for determination of HbA1C. The second blood sample was collected on plain tube, centrifuged at 3000 rpm/15 minutes after being allowed to clot. The serum samples were used for lipid profile, glucose, and insulin analysis.

The liver was removed, and then divided into two parts; the first part was kept in 10% neutral buffer formalin for histological investigation. While the second part was gathered on 1 ml of thiazole and frozen in liquid nitrogen at -80°C for later use in a gene expression investigation.

Biochemical analysis in serum

Following the manufacturer's instructions, commercial kits were used to help measure serum biochemical markers such as cholesterol (HDL and LDL/VLDL, USA), triacylglycerol (Quantification Abcam, USA), High-density lipoprotein-cholesterol (HDL-c) (Fluorometric Abcam, USA), LDL-c (Crystal Chem's Rat LDL, USA), glucose (Glucose assay kit MyBioSource, USA) and insulin (Insulin assay kit MyBioSource, USA). In order to determine the homeostasis model assessment for insulin resistance (HOMA-IR) index, which is calculated by multiplying blood glucose level (mg/dl) by insulin level (ng/ml) and then dividing on 405.

Measurement of Glycated haemoglobin (HbA1c)

DCA-2000 system (Ames, Bayer Diagnostics, Basingstoke, England) was used to assess HbA1c levels. The employed monoclonal antibody recognized a specific amino acid sequence (NH2-terminal) in the beta chain of glycated Hb. The binding of the antibody required both glucose and this particular sequence of amino acids. Latex-bead-bound monoclonal antibody was given in the absence of HbAlc and agglutinated by the second reagent. To prevent agglutination, glycated Hb acted as a competitor with the agglutinating agent for antibody binding sites. The absorbance at 531 nm was found to be higher than normal because of the agglutination reaction. HbAlc concentration was determined as a percentage of total Hb. HbA1c levels were measured in relation to total haemoglobin and the result was expressed as a percentage (Hsieh *et al*., 2013).

Gene expression

Following the protocols established by Khamis and his co-workers (Khamis *et al*., 2020), 500 ng of total RNA was reverse-transcribed to produce mRNA, while 10 ng were reverse-transcribed to produce miRNA using the TaqManTM Small RNA Assays according to the manufacturer's instructions. Assay design software (http://genomics.dote.hu:8080/ mirnadesigntool) (Czimmerer *et al*., 2013) was used to create the Stem-loop RT and miRNA specific primers, as well as the universal reverse primer (Table 1). Maxima SYBR Green/Rox qPCR Master Mix (2X) was used in the real-time PCR, and the procedures were carried out as per the manufacturer's recommendations. Fold change was reported as $2^{-\Delta\Delta CT}$ relative to control for each gene's expression level after normalization to either housekeeping GADPH (for mRNA) or U6 (for miRNA) (Livak and Schmittgen, 2001).

Histopathological investigations

Tissue samples were fixed in 10% neutral buffered formalin for 48 hours, and then washed overnight in running water. After washing, the samples were dehydrated with ethyl alcohol at strengths between 70% and 100%. The samples were kept in ethyl alcohol for 12 hours before being rinsed for 2 hours with xylol.

Table 1. Primers sequences for the real time PCR.

Gene	Sequence	Accession number
SMO	F 5′-TTCCTCATCCGAGGGGTCAT-3’ R 5’- ATTGATCTTGCTGGCTGCCT-3’	NM_012807.1
GLI1	F 5′-CCTCCACCCCAGTATCTCCA-3’ R 5’-ACAATTCCTGCTGCGACTGA-3’	NM_001191910.1
Ptch 1	F 5′-TCCCCTCCTCCTCCTCTTTC-3’ R 5’-CTTGTTCTCCTCACCGACCC-3’	NM_053566.3
Hhip	F 5′- GCTCTTTGGTCCTGATGGCT-3’ R 5’-GCTGGTTGGTGCTGTTGAAG-3’	NM_001191817.1
SHH	F 5′-GTAACGCTACGAGAGGAGGC-3’ R 5’- GAGCACCCGGTTGATGAGAA-3’	NM_017221.1
TLR4	F 5′-ACTGGGTGAGAAACGAGCTG-3’ R 5’-CAGCAATGGCTACACCAGGA-3’	NM_019178.2
NF-kB	F 5′-GCACCCCACCATCAAGATCA-3’ R 5’-CACACTGGATCCCCAGGTTC-3’	NM_199267.2
GADPH	F 5′-GCATCTTCTTGTGCAGTGCC-3’ R 5’-TACGGCCAAATCCGTTCACA-3’	NM_017008.4
miRNA 26a	R 5’-AATCGGCGTTCAAGTAATCCAG-3’ R 5’- GTCGTATCCAGTGCAGGGT-3’ Stem-loop primer;GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCCTA	
U6	R 5’- GCTCGCTTCGGCAGCACA-3’ R 5’- GAGGTATTCGCACCAGAGGA-3’ Stem-loop primer; AACGCTTCACGAATTTGCGTG	

Then samples were put in a crucible containing molten paraffin. Tissue was paraffin-embedded and sectioned to a thickness of 5 microns before being examined under the microscope. H&E-stain used for histopathological examination.

Statistical analysis

Standard Error of Mean was used to report the results. Two-way analysis of variance (ANOVA) with Duncan’s multiple test for post hoc comparisons was used to analyze the effect of the groups on the various biochemical markers. Statistical significance was less than 0.05. SPSS 25 and GraphPad Prism 8 were used to conduct statistical analyses and generate charts.

RESULTS

Effects of garlic oil and leek powder on body weight and BMI in HFD-induced obesity

Within the 10-week study period, rats in the HFD group demonstrated a significant increase in body weight and BMI (Figure 1A, B). The body weight of rats and BMI in the treatment groups and HFD group was significantly higher than control group and prophylactic groups at 10 weeks. At 18 weeks, the HFD group showed the largest weight gain and BMI, and it was significantly changed than other groups.

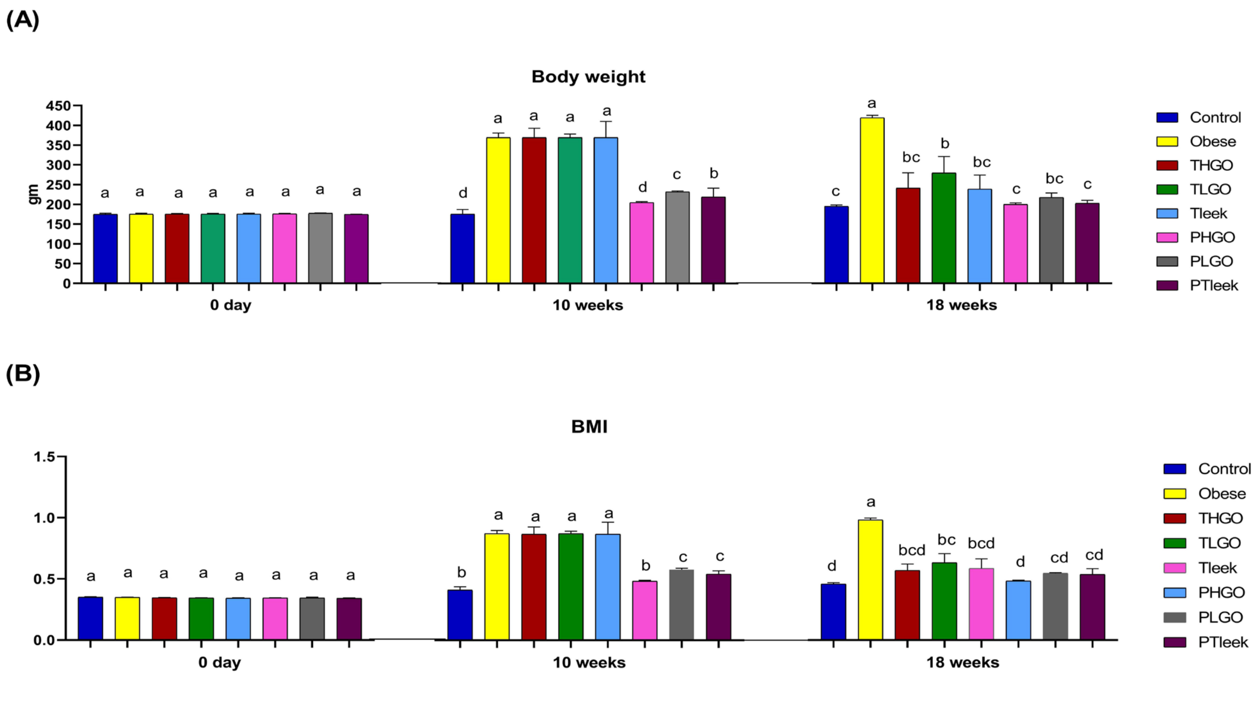

Fig. 1. Effects of garlic oil and leek powder on body weight (gm) and BMI in HFD-induced obesity.

Effects of garlic oil and leek powder in some biochemical parameters in HFD-induced obesity

The result of the present study in Figure 2 revealed that HbA1c, insulin, and HOMA-IR levels were the highest ($p < 0.05$) in obese groups in comparison with other groups. While their levels were significantly ($p < 0.05$) improved in the prophylactic groups (PHGO, PLGO, PTleek) and treatment groups (THGO, TLGO, Tleek) in comparison with obese groups. HbA1c levels were non-significantly differ in prophylactic groups (PHGO, PLGO, PTleek) and treatment groups (THGO, TLGO, Tleek). Insulin levels were non-significantly differ in between prophylactic groups (PHGO, PLGO, PTleek) each other. On the other hand, prophylactic groups showed significant improvement than the treatment groups (THGO, TLGO, Tleek). HOMA-IR were non-significantly differ in between prophylactic groups (PHGO, PLGO, PTleek) each other, and treatment groups (THGO, TLGO, Tleek) were non-significantly differ in between each other.

Effects of garlic oil and leek powder in lipid profile in HFD-induced obesity

Figure 3 shows that cholesterol, triacylglycerol, LDL-c and VLDL-c levels were the highest in obese groups in comparison with other groups. While their levels were significantly ($p < 0.05$) improved in the prophylactic groups (PHGO, PLGO, PTleek) and treatment groups (THGO, TLGO, Tleek) in comparison with obese groups. HDL-c levels were non-significantly differ in

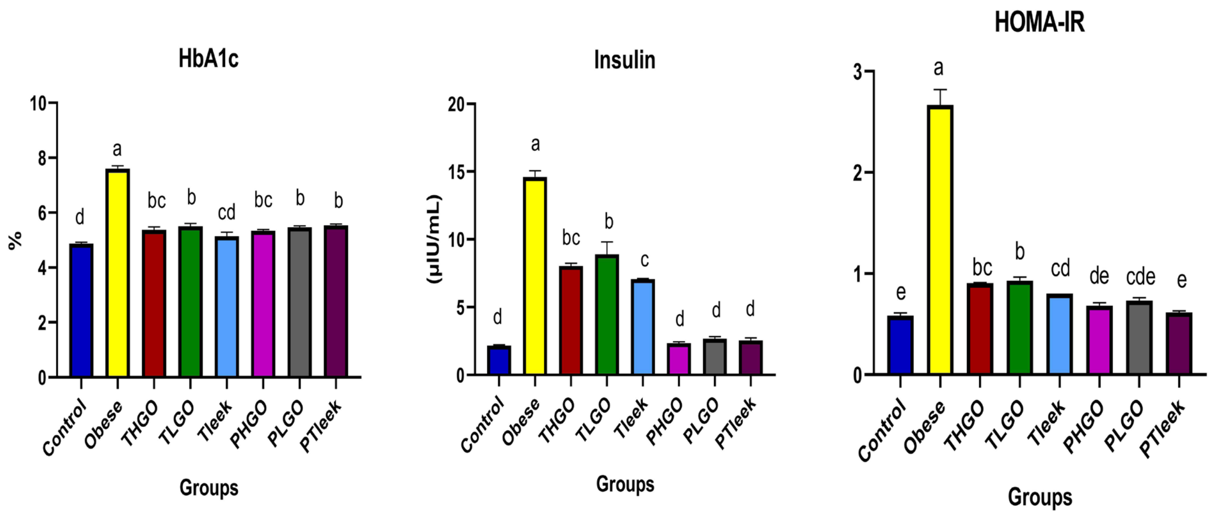

Fig. 2. Effects of garlic oil and leek powder in the levels of some biochemical parameters in HFD-induced obesity.

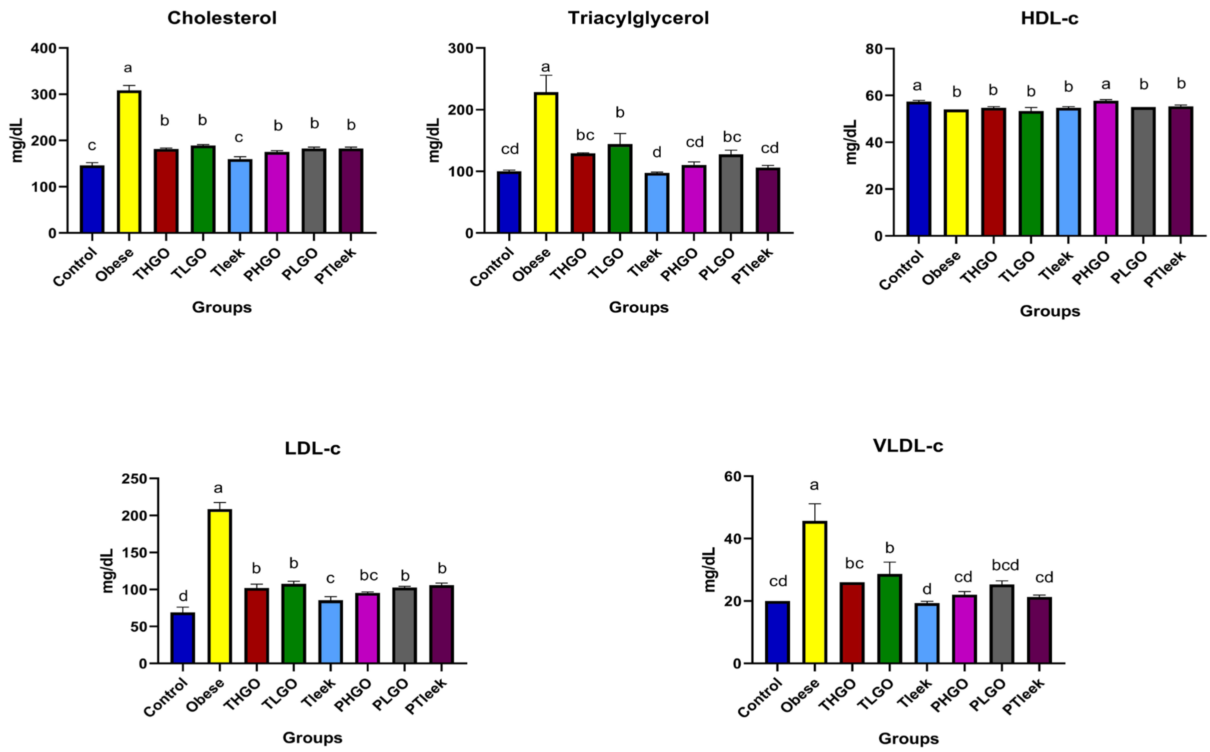

Fig. 3. Effects of garlic oil and leek powder in lipid profile in HFD-induced obesity.

obese groups, prophylactic groups (PLGO, PTleek) and treatment groups (THGO, TLGO, Tleek). HDL-c levels were non-significantly differ in PHGO group and control negative group.

Effects of garlic oil and leek powder in the levels of expression of some genes in HFD-induced obesity in tissue homogenate

The mRNA expression of *SMO*, *GLI1*, and *Ptch1* in Figure 4 demonstrated the highest levels in obese groups and significantly downregulated in the prophylactic groups (PHGO, PLGO, PTleek) and treatment groups (THGO, TLGO, Tleek) ($p < 0.05$). The lowest levels were in control negative groups. On the other hand, Hhip-1 activity was the lowest level in obese groups and significantly up-regulated in the prophylactic groups (PHGO, PLGO, PTleek) and treatment groups (THGO, TLGO, Tleek). The highest level was in control negative group ($p < 0.05$).

The expression of *SHH*, *TLR 4*, and *NF-kB* in Figure 5 revealed their activities were at the highest levels in obese groups and significantly downregulated in the prophylactic groups (PHGO, PLGO, PTleek) and treatment groups (THGO, TLGO, Tleek). The

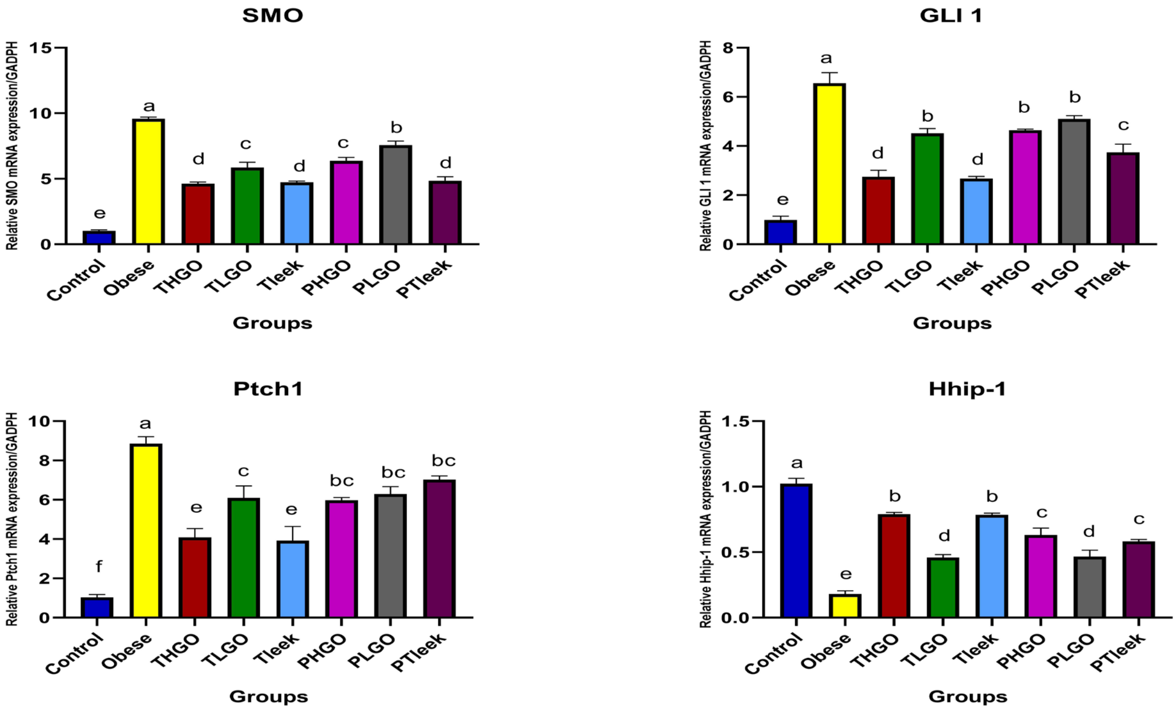

Fig. 4. Effects of garlic oil and leek powder in the mRNA expression of some genes in HFD-induced obesity.

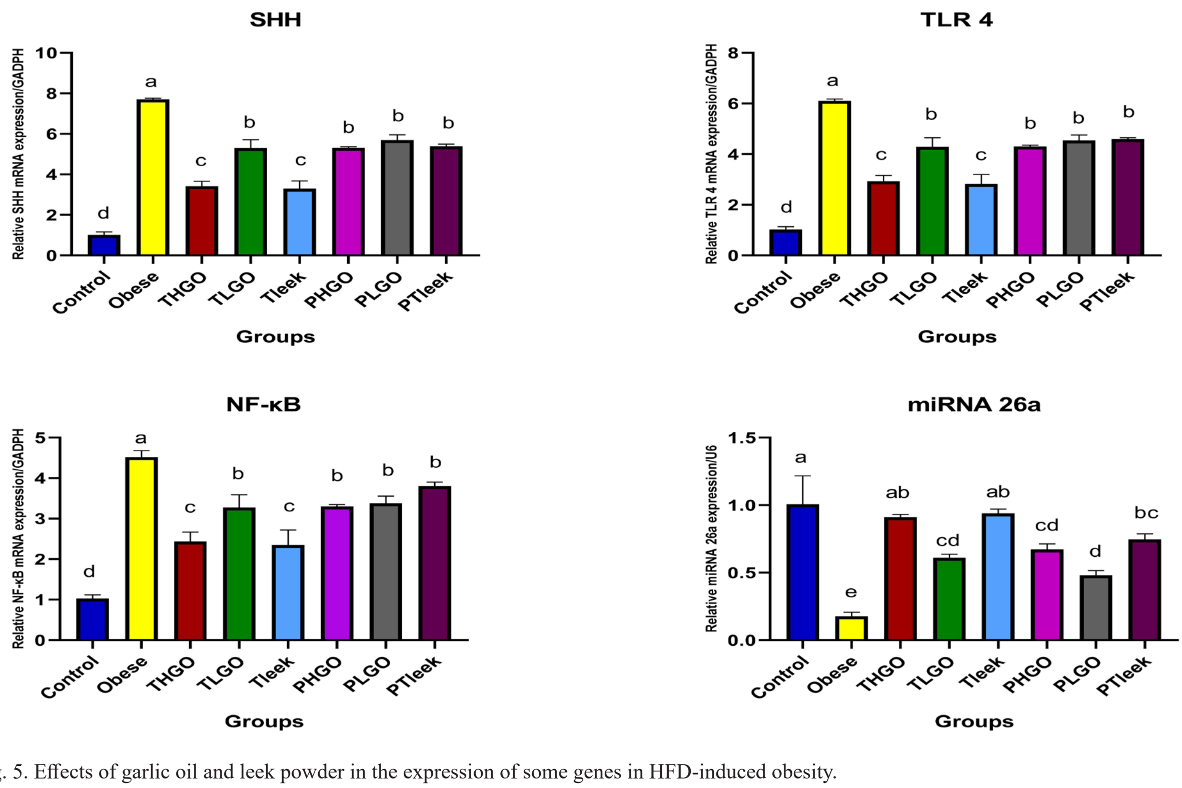

Fig. 5. Effects of garlic oil and leek powder in the expression of some genes in HFD-induced obesity.

lowest levels were in control negative groups. On the other hand, *miRNA 26a* activity was the lowest level in obese groups and significantly upregulated in the prophylactic groups (PHGO, PLGO, PTleek) and treatment groups (THGO, TLGO, Tleek). The highest level was in control negative group.

Histopathological findings

The liver tissue from a control rat is shown in normal structure in Figure 6A. The liver tissue of obese group showing significant fatty degeneration of most of the hepatocytes (Figure 6B). Garlic oil and leek powder in the prophylactic and treatments groups showed an improvement in the damage induced by HDF (Figure 6C-H).

DISCUSSION

The alarming rise in diabetes risk factors associated with obesity have caught the attention in emerging countries. Obesity lowers HDL-C levels and raises TG levels in the blood. A high-fat diet is a key risk factor for obesity because it can encourage a positive energy balance and boost visceral fat deposition (Khalil *et al*., 2018). Some compounds in garlic have been linked to success in treating obesity and high cholesterol (Bisen and Emerald, 2016).

Two of the most valuable veggies are garlic, and leeks. They include a plethora of nutrients, including protein, fibre, polyphenols, and flavonoids. In addition to phosphate, calcium, and carbs, it also contains protein. The anthelmintic, antioxidant, anti-inflammatory, and antiseptic properties of plants in the galic family are well documented. Garlic, and leek all have varying levels of polyphenolic chemicals due to harvest, storage, and environmental factors (Lenkova *et al*., 2016).

In this study, the impacts of garlic oil and leek powder on hyperlipidemia produced by the ingestion of a HDF were assessed in animals. Garlic oil and leek powder were tested for their ability to reduce obesity indicators in rats fed a HDF. Rats fed the garlic oil and leek powder diets had significantly decreased lipid profile and insulin, HbA1C indices compared to those on the HFD diet. These findings point to the anti-obesity effects garlic oil and leek powder. Garlic extract has been hypothesized to have potential anti-obesity benefits (Kim and Kim, 2011).

In the present study, compared to the HFD group, those who consumed garlic oil and leek powder had a better lipid profile, with reduced serum cholesterol, triacylglycerol, LDL-c and VLDL-c levels. Our results are in line with Mahmoodi *et al*. declared that a diet rich in garlic can reduce LDL cholesterol and raise HDL-C levels in hyperlipidemic (Mahmoodi *et al*., 2006), animal, and coronary heart disease (Kwon *et al*., 2003) patients. Last but not least, the obtained data showed that the effective dose of garlic oil was the highest dose, rather than the lowest dose.

Research from this study showed that the beneficial physiological effects of garlic oil and leek powder may be obtained at non-toxic levels. Histological studies of the livers of rats given an HFD showed severe fatty degeneration. In addition, there was a significant improvement in the garlic oil and leek powder groups compared to the HFD group. As a result, garlic oil and leek powder may have significant effects on lipid metabolism and body fat.

Garlic is renewable resources having great application value, and the current study showed that it had anti-obesity and hypolipidemic actions in the rodent. It has great potential as foods, but it also has many other uses in medicine, food additives, feed, and other areas (Sajitha *et al*., 2016; Şen Tanrıkulu *et al*., 2017).

By measuring blood glucose, insulin, and oxidative stress in rats given fructose, Padiya *et al*. (2011), demonstrated that homogenized raw garlic is helpful in enhancing insulin sensitivity. Supplementing with garlic powder has been shown by Sangouni *et al*. (2020), to reduce insulin resistance and oxidative stress, two major contributors to fatty liver disease in people with NAFLD. Mice fed a diet supplemented with aged garlic extract also showed reduced insulin resistance, as recently found (Ettehad-Marvasti *et al*., 2022). Allicin and dialyl sulphide are two of the active chemicals in garlic that contribute to its hypoglycemic action (Madkor *et al*., 2011). Considering that obesity is a major contributor to insulin resistance (Verkouter *et al*., 2019), it was demonstrated that two months of oral administration of garlic extract resulted in both a considerable reduction in body weight and an improvement in insulin sensitivity. Thus, it's possible that the participants' decreased body mass contributed to the observed enhancement of insulin sensitivity (Ettehad-Marvasti *et al*., 2022).

In addition, several animal studies have shown that when mice were fed a high-fat diet and allowed to gain weight, exogenous H2S treatment resulted in greater insulin sensitivity and improved glucose tolerance (Geng *et al*., 2013). Depletion of endogenous H2S production prevented high fat diet-induced fat mass in mice and fruit flies, although supplementation with H2S donors was sufficient to increase fat mass formation (Yang *et al*., 2018).

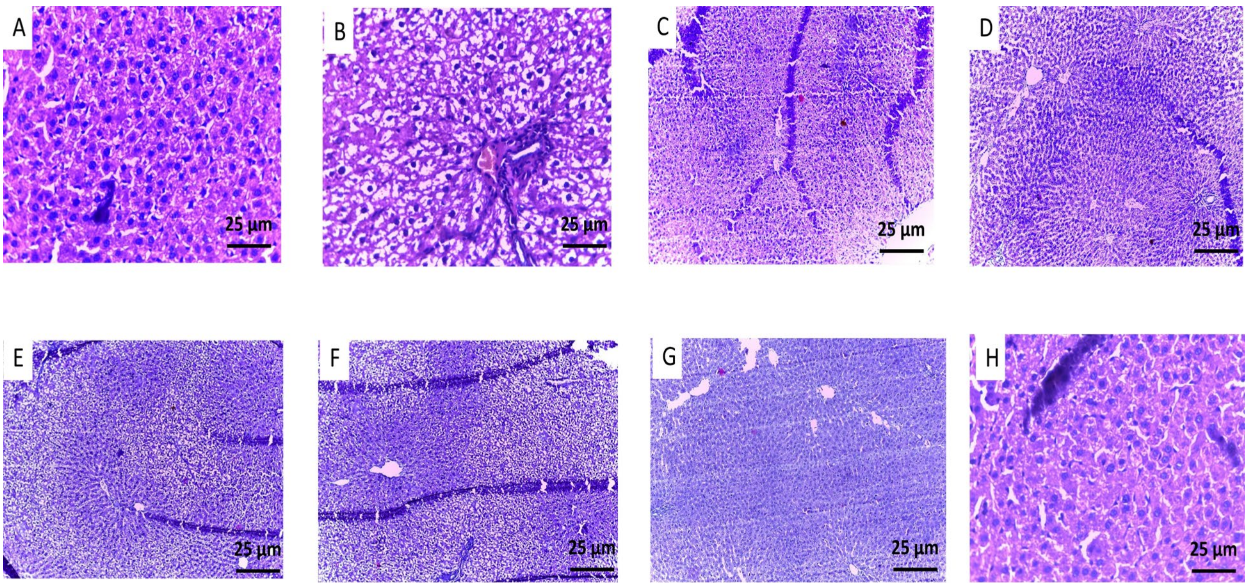

Fig. 6. Histopathological results in liver. (A) liver tissue of control rat, H&E, X 40. (B) liver tissue of obese rat, H&E, X 40. (C) liver tissue of THGO treated rat, H&E, X 40. (D) liver tissue of TLGO treated rat, H&E, X 40. (E) liver tissue of Tleek treated rat, H&E, X 40. (F) liver tissue of PHGO treated, H&E, X 40. (G) liver tissue of PLGO treated rat, H&E, X 40. (H) liver tissue of PTleek treated rat rats, H&E, X 40.

This study revealed a reduction in the gene expression of *Ptch1*, *SMO*, *GLI1*, and *SHH* by garlic oil and leak powder. On the other hand, a significant upregulation was found in the expression of Hhip-1, the following studies illustrated these results.

The Hh signalling pathway regulates tissue growth and homeostasis, and it has been conserved throughout evolution. *Ptch1* is a receptor for the Hh ligands, and upon binding, *Ptch1* releases its inhibition on *SMO*othened (*SMO*), a seven-pass transmembrane protein, allowing the GLI family of transcription factors to activate transcription (Xu *et al*., 2018). Insulin resistance is increased in the liver tissues in people with type 2 diabetes. The upstream regulation of the Hh pathway is affected by the overexpression of *SMO* and Patched 1, which contributes to this resistance (Lin *et al*., 2019).

The mRNA and protein expression of the Hh pathway transcription factor GLI1 in AsPC-1 PC cells was demonstrated to be suppressed by one of garlic derivatives (DATS) at 16 and 24 hours. These results imply that DATS may ultimately result in Hh pathway suppression because *GLI1* is the downstream effector of the pathway. Recent clinical studies of the *SMO* inhibitor IPI-926 in combination with gemcitabine dramatically reduced median survival in metastatic PC patients compared with gemcitabine treatment alone, therefore these findings are relevant when thinking about the chemopreventive application of DATS. The observation that *GLI1* expression is suppressed in AsPC-1 cells provides support for the concept that DATS down regulates the Hh pathway (Ko, 2015; Puccinelli, 2016).

Data from the current study revealed a reduction in the expression of *TLR 4*, and *NF-kB* by garlic oil and leek powder. Mechanistic and therapeutic evidence strongly suggests that *NF-kB* is involved in the inflammation and insulin resistance that characterize obesity. NF-κB is a molecular link to obesity, inflammation, and insulin resistance (Kim, 2012). Obesity-related inflammatory responses are thought to be mediated primarily by TLR4, a member of the natural immune receptor family. It was reported that TLR4 signalling pathway activation by triglycerides increases expression of inflammatory markers in hepatocytes. This finding suggests a link between obesity-induced reduced inflammation and TLR4 expression (Jiang *et al*., 2021).

The present study showed a significant upregulation in the *miRNA 26a* expression which is induced by garlic oil and leek powder. The miR-26a plays a crucial role as a tumour suppressor and in the liver—the primary organ responsible for glucose and lipid homeostasis. Insulin signalling, fatty acid metabolism, and gluconeogenesis are all regulated by miRNA-26a, which acts as a target on the corresponding genes. In together, these results highlight the importance of miRNA-26a in liver metabolism and indicate that it may be a useful therapeutic target for preventing type 2 diabetes in humans with diet-induced obesity by increasing insulin sensitivity and decreasing lipogenesis and gluconeogenesis (Fu *et al*., 2015). Almost scant studies cover the Hh pathway components and *miRNA 26a* in obesity and its relation to garlic oil and leek powder.

CONCLUSION

Both garlic oil and leek powder demonstrate potential an anti-obesity drug. Inhibiting the Hh pathway is how garlic oil and leek powder can lower blood HOMA-IR, insulin levels, lipid profile, and liver structural damage in obese rats. The effects of garlic oil and leek powder on Hh signalling pathways and *miRNA 26a* in obesity have not previously been investigated, making this study potentially the first of its kind. The incorporation of H2S donors into treatment techniques may open up new possibilities for combating chronic diseases like obesity.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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