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Research article

# Modulation of adiposity and adipocyte inflammation by methanol extracts *of Alpinia calcarata* leaf in high-fat-diet induced-obese mice: Involvement of COX-2 and PPAR-γ

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ABSTRACT

Obesity is a worldwide problem linked to several lifestyle disorders like diabetes, hypertension, heart failure, dyslipidaemias, asthma, etc. Finding a cure for obesity and its consequences is essential. *Alpinia calcarata*, a plant from the Zingiberaceae family, has been reported for several medicinal properties. The current study aimed to check out the role of *Alpinia calcarata* leaf in decreasing adiposity and adipocyte inflammation in high-fat diet-induced obese mice and understand the molecular principles underlying this occurrence. An *in-silico* test was done with more abounded compounds of *Alpinia calcarata* with adiposity and inflammatory genes. Moreover, methanol extract of *Alpinia calcarata* leaves were utilized to confirm the *in-silico* data *in-vivo*. High-fat diet induced-obese mice were treated with the extract at 200mg/kg-body weight dose. Body weight, organ weight, fat accumulation, serum cholesterol, serum triglyceride level, and liver function test were monitored as function of obesity. Alteration in the expression of IL-6, COX-2, MCP-1, PPARγ, TNFα, and GLUT-4 at transcript level were also studied. Our *in-vivo* results indicated that the plant extracts significantly ($p < 0.05$) decreased weight and accumulation of abdominal fat which was followed by a considerable reduction in total cholesterol and triglyceride levels. In agreement with the *in-silico* data, the extract was capable to reduce the mRNA expression of IL-6, COX-2, MCP-1, PPARγ, TNFα, and GLUT-4 which were consistent with the biochemical evidence; demonstrating the extract's capacity to attenuate adiposity and adipocyte inflammation. Taking it all together, it is noteworthy to report this novel function of *A. calcarata* leaf in reducing adiposity and adipocyte inflammation.

## 1. Introduction

In recent decades, the frequency of obesity and overweight has risen considerably [1]. Obesity and overweight have been connected to a variety of non-communicable diseases (NCDs), notably type 2 diabetes (diabetes mellitus), stroke, ischemia, hypertension, and many more. Moreover, Inflammatory markers including CRP, IL-6, and TNFα are higher in obesity, which has been classified as a

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**List of abbreviations:**

| | |
|---|---|
| COX-2 | Cyclooxygenase-2 |
| CRP | C-Reactive Protein |
| GLUT-4 | Glucose transporter-4 |
| HFD | High-fat diet |
| IL-1α | Interleukin-1 alpha |
| IL-1β | Interleukin-1 beta |
| IL-6 | Interleukin-6 |
| LPS | Lipopolysaccharides |
| MCP-1 | Monocyte Chemotactic Protein-1 |
| NCDs | Non-Communicable Diseases |
| ND | Normal diets |
| PPARγ | Peroxisome proliferator-activated receptor-gamma |
| SEM | Standard Error of the mean |
| SGOT | Serum Glutamic-Oxaloacetic Transaminase |
| SGPT | Serum Glutamate Pyruvic Transaminase |
| TC | Total Cholesterol |
| TF | Transcription factor |

low-grade chronic inflammatory condition [2]. Numerous studies show that obesity stimulates inflammation, and this type of inflammation affects a variety of organs, including the liver, brain, pancreas, skeletal muscle, and heart [3].

Certain long-chain fatty acids and some eicosanoids serve as natural ligands to activate PPARγ, the master regulator of adipogenesis. Previously, we showed that mature cultured adipocytes may generate pro-adipogenic prostaglandins via the cyclooxygenase (COX) route and that activating PPARγ helps the adipogenesis process. COX-2-derived prostaglandins are involved in obesity-associated metabolic syndrome and play a role in energy regulation under different pathophysiological conditions. Moreover, COX-2 the inducible gene is primarily involved in the regulation of inflammation [4]. Blocking the COX pathway with conventional non-steroidal anti-inflammatory drugs has been recognized to be associated with different side effects including gastrointestinal ulcers, bleeding, and renal disorders [5].

Lifestyle and dietary modification and increased physical activity are very common approaches to reducing obesity and overweight [6]. A broad range of natural compounds has been identified with anti-obesity properties, having lower toxic effects and wide-ranging synergistic effects [7]. As a result, using plants and their constituents may be a useful method for the management of obesity and related illnesses. Very recently, we reported that *Musa acuminate* seed extract can reduce obesity and related complications through the suppression of PPARγ [8].

The rhizomatous plant *Alpinia calcarata* Roscoe (Zingiberaceae) is found extensively throughout Bangladesh, India, and Sri Lanka. This herb is mostly utilized by Bangladesh's tribal populations (Murong, Chakma, and Tanchangya) for a variety of diseases, including bronchitis, colds, respiratory problems, asthma, and arthritis [9]. The rhizomes of this plant have some medicinal properties including antifungal, antibacterial, aphrodisiac, anthelmintic, antioxidant, gastroprotective, antidiabetic, etc [10] whereas the leaves of *A. calcarata* possess analgesic, anxiolytic, sedative effect, antioxidant, antibacterial potentials, etc [9,11]. Among other constituents of *A. calcarata* leaf, 1,8 cineole, and carotol have been reported for anti-inflammatory activity [12]. Based on the existing knowledge from the literature, there are no reports on the use of *A. calcarata* leaves in adiposity and adipocyte inflammation. Taking it all together, we wanted to experimentally demonstrate that *Alpinia calcarata* Roscoe leaf can reduce adiposity and adipocyte inflammation in high-fat diet-induced obese mice for the first time and also wanted to look into the molecular mechanisms behind this phenomenon.

## 2. Materials and methods

### 2.1. In silico study

Compounds of *Alpinia calcarata* were identified by literature review. The major components of *Alpinia calcarata* found in Bangladesh are 1,8-cineole (28.48 %), Camphor (21.40 %), Methyl cinnamate (13.35 %), Carotol (6.53 %), β –Pinene (6.39 %), etc

**Table 1**
Compounds of *Alpinia calcarata* and related receptors.

| Compounds | PubChem ID | Receptors | Uniprot ID |
|---|---|---|---|
| Carotol | 442347 | COX-2 | Q05769 |
| 1,8 cineole | 2758 | PPARγ | P37238 |
| β-pinene | 14896 | IL-6 | P08505 |
| Camphor | 2537 | GLUT-4 | P14142 |
| Methyl cinnamate | 637520 | | |

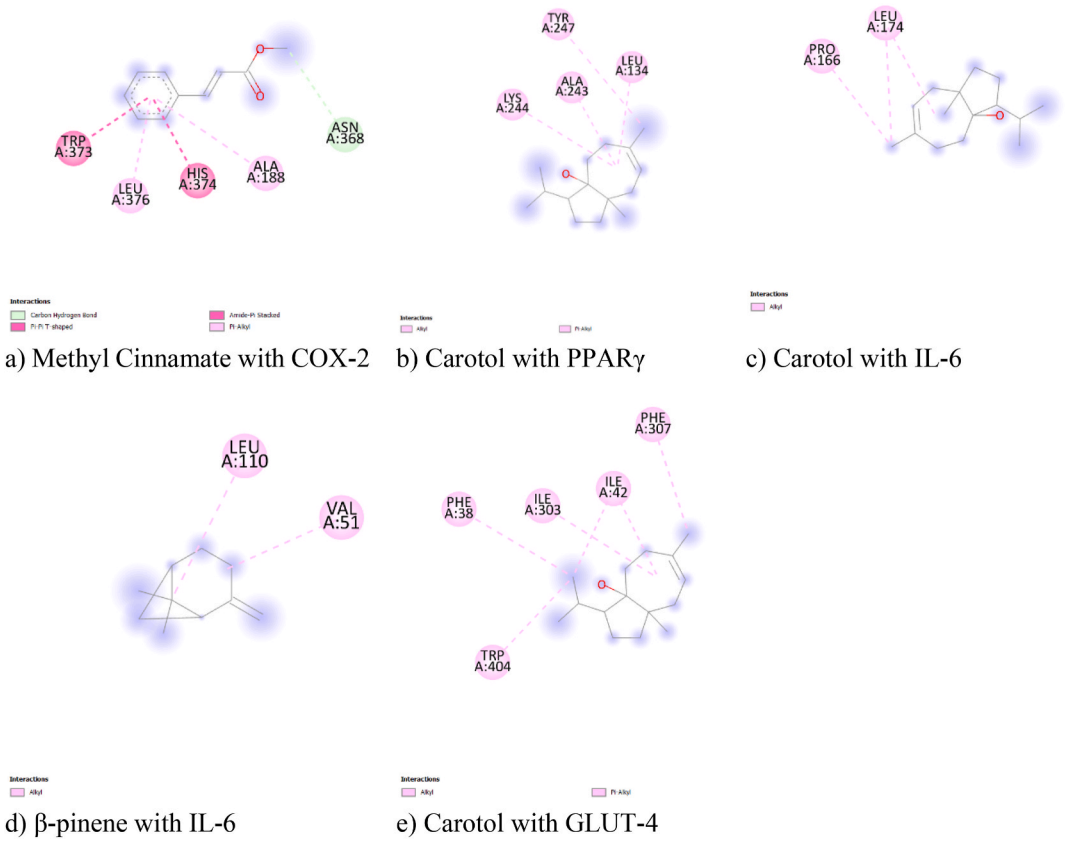


**Fig. 1.** 2D interaction of highest binding affinity of proteins with plants compounds. (a) Methyl Cinnamate with COX-2, (b) Carotol with PPARγ, (c) Carotol with IL-6, (d) β-pinene with IL-6, (e) Carotol with GLUT-4.

[13]. Then these compounds were downloaded from the website PubChem (https://pubchem.ncbi.nlm.nih.gov/). The compounds with their PubChem ID are listed in Table 1. Different proteins of adipogenesis and inflammation were selected and then these genes of *mus musculus* were identified from the website Uniprot (https://www.uniprot.org/). With Uniprot ID, the 3D structures of proteins were downloaded from Alphafold Protein Structure Database [14]. Though the proteins were fully prepared, but we also visualize these through Discovery Studio 2016. The targeted proteins are listed on Table 1. Molecular docking of selected compounds and proteins was done by PyRx virtual screening tool (version 0.9.9) and then the final structure was visualized by Discovery Studio Visualizer 2016 and collect the bond distance, binding type, or category etc. The pharmacokinetic profile is essential for a substance to use as a drug. The absorption, distribution, metabolism, excretion and toxicity were virtually measured by different websites like; Swiss ADME (www.swissadme.ch/), pkCSM (biosig.lab.uq.edu.au/pkcsm/) for pharmacokinetic study and ProTox II (tox-new.charite.de/protox_II/index.php?site=home) for toxicity study. These servers forecast different essential parameters like; water solubility, skin permeability, CaCO2 permeability, intestinal absorption, BBB permeability, clearance, toxicity etc. The canonical SMILE format of each compound is an input format that obtained from PubChem.

### 2.2. Plant extract preparation

The desired plant leaves of *A. calcarata* were harvested from botanical garden in Dhaka, Bangladesh. The plant was validated by the Dhaka National Herbarium and the accession number is DACB-73889. *A. calcarata* leaves had been sorted, cleaned, and dried in air at ambient temperature for around weeks. The leaves were crushed by blender and stored in a tightly sealed container in a cool and dry environment till needed. Amounting 400 gm of crushed plant material, 1500 ml of 80 % methanol was added to a clean, flat-bottomed glass container. The mixture was allowed to sit at room temperature for 15 days while being intermittently shaken and stirred.

After filtration, the extracts had been evaporated by using a rotary evaporator under decreased pressure at $40^0$C. (RE-EV311-V, LabTeck S.R.L, Italy). The raw extracts were weighed after being freeze-dried and stored in desiccators until needed.

### 2.3. Experimental animals and diet

A total of fifteen healthy adult Swiss albino mice (weighing 22–25 g) were employed in this study. Mice were purchased from the

**Table 2**
Binding score of compounds of *Alpinia calcarata* with receptors.

| Compounds | Highest binding affinity with receptors | | | |
|---|---|---|---|---|
| | COX-2 | PPARγ | IL-6 | GLUT-4 |
| **Carotol** | −6.1 | −6.4 | −5.7 | −6.9 |
| **1,8 cineole** | −4.9 | −5.2 | −4.8 | −5.9 |
| **β-pinene** | −5.2 | −5.4 | −5.7 | −5.8 |
| **Camphor** | −5.3 | −5.7 | −5.1 | −6.2 |
| **Methyl cinnamate** | −6.5 | −6.3 | −5.6 | −5.7 |

**Table 3**
Molecular docking analysis of highest binding interacting compounds with proteins.

| Compound | Binding energy | Interacting Amino Acid | Distance | Category | Type |
|---|---|---|---|---|---|
| COX-2 | | | | | |
| **Methyl cinnamate** | −6.5 | ASN368 | 3.79069 | Hydrogen Bond | Carbon Hydrogen |
| | | HIS374 | 5.28779 | Hydrophobic | Pi-Pi T-shaped |
| | | TRP373: HIS374 | 3.79569 | | Amide-Pi Stacked |
| | | ALA188 | 5.29754 | | Pi-Alkyl |
| | | LEU376 | 5.20156 | | |
| **PPARγ** | | | | | |
| **Carotol** | −6.4 | LEU134 | 4.50648 | Hydrophobic | Alkyl |
| | | ALA243 | 4.91003 | | |
| | | LYS244 | 5.02902 | | |
| | | TYR247 | 4.54861 | | Pi-Alkyl |
| **IL-6** | | | | | |
| **Carotol** | −5.7 | LEU174 | 4.53098 | Hydrophobic | Alkyl |
| | | PRO166 | 4.19263 | | |
| | | LEU174 | 5.21821 | | |
| **β-pinene** | −5.7 | VAL51 | 4.11289 | Hydrophobic | Alkyl |
| | | LEU110 | 5.15032 | | |
| **GLUT-4** | | | | | |
| **Carotol** | −6.9 | ILE42 | 5.35864 | Hydrophobic | Alkyl |
| | | ILE303 | 5.39559 | | |
| | | ILE42 | 5.006 | | |
| | | PHE38 | 4.61212 | | Pi-Alkyl |
| | | PHE307 | 4.00324 | | |
| | | TRP404 | 5.36281 | | |

animal house at Jahangirnagar University, Bangladesh. Mice were maintained in different metal boxes under-regulated settings of 12-h Dark-Light cycles and consistent environmental and dietary parameters. The special nipple offered unlimited access to fresh and pure drinking water twice a day. The boxes were periodically cleaned 2–3 times per week. The trials were conducted after a 7-day acclimatization period. All animal trials were carried out at the Department of Pharmacy, Noakhali Science and Technology University, Bangladesh. The Institutional Ethical Committee of Noakhali Science and Technology University examined and recognized the ethical issues (NSTU/SCI/EC/2022/106).

### *2.4. Experimental design and animal grouping*

High fat diet (HFD) utilized in our previous study [8] was given to the mice to induced obesity. The fifteen mice are first separated into two categories: the high-fat diet (HFD), which comprises ten mice who consumed fat containing diets for a month until gaining weight, and the normal diets (ND) group, which consists of five mice who were provided normal diets during this period. After four weeks, the HFD group was subsequently separated into two groups, each with five mice for treatment with the extract. One remained with the previous name HFD group. The remaining one was renamed: High-fat diet plus extract. A dose of 200mg/kg-body weight of mice of plant extract was administered orally for six weeks. All two groups were kept on their regular high-fat diets, whereas the ND group was kept on their normal diet. After the experiment, the animals were dissected, and blood samples were obtained for biochemical evaluation.

### *2.5. Biochemical analysis*

An automated biochemical analyzer (Mindray BA-88A, China) with a widely accessible kit (Cromatest, Linear Chemical SL) was used to assess total cholesterol (TC), and serum triglycerides (TG); also, the liver function tests like Serum Glutamate Pyruvate Transaminase (SGPT) and Serum Glutamic Oxaloacetic Transaminase (SGOT) were measured by using a commercial kit (Human Diagnostics, Germany) used in our previous study [8].

**Table 4**
ADMET analysis.

| Carotol | 222.36 | 3.92 | 70.46 | −4.146 | 1.544 | 94.803 | −2.169 | 0.604 | 1.091 | No | 0 | 5 | 4300 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1,8 cineole** | 154.25 | 2.7441 | 47.12 | −2.63 | 1.485 | 96.505 | −2.437 | 0.368 | 1.009 | No | 0 | 5 | 2480 |
| **β-pinene** | 136.23 | 2.9987 | 45.22 | −4.191 | 1.385 | 95.525 | −1.653 | 0.818 | 0.03 | No | 1 | 5 | 4700 |
| **Camphor** | 152.23 | 2.4017 | 45.64 | −2.895 | 1.499 | 95.965 | −2.002 | 0.612 | 0.109 | No | 0 | 4 | 775 |
| **Methyl cinnamate** | 162.19 | 1.8728 | 47.43 | −2.132 | 1.442 | 97.453 | −2.102 | 0.238 | 0.814 | No | 0 | 5 | 2610 |
| **Compound** | Molecular weight (gm/mol) | Log P | Molar Refractivity | Water solubility (log mol/L) | Caco2 permeability (log Papp in $10^{-6}$ cm/s) | Intestinal absorption (human) (% absorbed) | Skin Permeability (Log Kp) | BBB permeability (Log BB) | Total Clearance (log ml/min/kg) | Hepatotoxicity | Lipinski violation | Predicted toxicity class | Predicted LD50 (mg/kg) |

**Table 5**
List of primers with sequence.

| Gene | | Sequence |
|---|---|---|
| **PPARγ** | **S** | 5′ CTTCGTGATGACTGCCTAT 3′ |
| | **AS** | 5′ GGGTCAGCTCTTGTGAATGGA 3′ |
| **COX-2** | **S** | 5′ CCACTTCAAGGGAGTCTGGA 3′ |
| | **AS** | 5′ AGTCATCTGCTACGGGAGGA 3′ |
| **MCP-1** | **S** | 5′ TCAGCCAGATGCAGTTAACGC 3′ |
| | **AS** | 5′ CTGTCACACTGGTCACTCCTA 3′ |
| **TNFα** | **S** | 5′ GCCTCTTCTCATTCCTGCTTG 3′ |
| | **AS** | 5′ CTGATGAGAGGGAGGCCATT 3′ |
| **GLUT-4** | **S** | 5′ GGATTCCATCCCACAAGGCA 3′ |
| | **AS** | 5′ CCAACACGGCCAAGACATTG 3′ |
| **IL-6** | **S** | 5′ AACGATGATGCACTTGCAGA 3′ |
| | **AS** | 5′ GGTACTCCAGAAGACCAGAGGA 3′ |
| **β-actin** | **S** | 5′ GTTTGAGACCTTCAACACCCC 3′ |
| | **AS** | 5′ GGAGGAGCAATGATCTTGATC 3′ |

### 2.6. Extraction of mRNA from adipose tissue and qPCR analysis

As in our earlier investigation, the Trizol (Invitrogen) kit used to extract mRNA from white adipose tissue (Visceral fat) [8]. A Colibri Micro-Volume Spectrometer was used to measure the amounts of mRNA (Titertek-Berthold, Germany). Following the manufacturer's instructions, Protoscript-II (BioLabs Inc. New England, CAT #E6560S) was used to synthesize cDNA from mRNA. GenBank Specific sequence primers (Table-5) were acquired from Macrogen Inc. (South Korea). The CFX96 Touch Real-Time PCR Detection System (Bio-Rad, US) was employed for 40 cycles of quantitative PCR using the Luna Universal qPCR Master Mix (BioLabs Inc. New England). The relative gene expression of mRNA was quantified using the ΔΔCT technique and then controlled for β-actin expression.

### 2.7. Statistical analysis

The student's t-test is used to calculate all the values after their means and standard errors of the means (SEM) have been calculated. GraphPad Prism program for Windows, version 8.00, was used for all analyses. (GraphPad Software, La Jolla, CA, USA).

## 3. Results

The highest binding score of the compound carotol with PPARγ, IL-6 and GLUT-4. In COX-2, methyl cinnamate shows the highest binding affinity compared with others. The 2D-interactions of these highest binding compounds are given in Fig. 1(a–e).

β-pinene have the same binding score like carotol with IL-6. The binding score of the selected compounds with adipogenic proteins are listed on Table 2. In molecular docking analysis of these highest binding affinity compounds with proteins, methyl cinnamate with COX-2 have one hydrogen bond and four hydrophobic bonds. Carotol have three, four and six hydrophobic bonds with IL-6, PPARγ and GLUT-4 respectively that are listed on Table 3 with interacting amino acid and distance.

In the ADMET analysis that are shown on Table 4, the compounds of *Alpinia calcarata* don't break the Lipinski rule except β-pinene. Also, these compounds have no hepatotoxic level. The predicted toxicity class was selected according to $LD_{50}$ value. All of them are on toxic class V ($2000< LD_{50} < 5000$), except camphore. Camphore is in toxic class IV ($300< LD_{50} < 2000$). The water solubility of these compounds is between −2.132 and −4.191. The CaCo2 permeability of all compounds are >0.90 and within the values between 1.385 and 1.544. All the compounds have the percentages of intestinal absorption are >90 %. Skin permeability is important factor to prepare transdermal drug product and low skin permeable value is less than −2.5; but all our selected compounds have the log Kp value of skin permeability is between −1.653 and −2.437. All these compound's BBB (Blood Brain Barrier) permeability is acceptable except methyl cinnamate which value is 0.238. All except methyl cinnamate BBB value is > 0.3 that means they can readily cross the blood brain barrier.

Fig. 2(a–e) describes the body weight gaining pattern, average body weight, food intake behaviour, energy intake, and abdominal fat weight of different groups of mice. In this study, the body weight, and abdominal fat were significantly increased in the high-fat diet compared to normal diet group. But treatment with the extract in the presence of high-fat diet values was significantly decreased. Food intake behaviour among the groups of mice were not changed significantly during the course of study Whereas mice on HFD received a significantly higher amount of calorie than normal fed mice and the extract did not affect the caloric intake behaviour. Effect of extract on other organ has been presented in Fig. 3(a–e).

The liver function and lipid profile tests of these different groups of mice are presented in Fig. 4(a–d). Serum triglyceride and total cholesterol was significantly increased in the high-fat diet group compared to normal diet group whereas the extract of interest was capable enough to rescue the phenomena significantly. The SGPT and SGOT values were increased in HFD group compared to the control, but the extract groups weren't changed.

Gene expressions of PPARγ, COX-2, GLUT-4, TNFα, MCP-1, and IL-6 of different mice groups are described in Fig. 5(a–f). Transcript levels of all the genes studied here were significantly increased in the HFD group of mice than in the normal diet group. In agreement with the biochemical data, significantly reduced mRNA expression was observed in animals fed with the extract together with high fat.

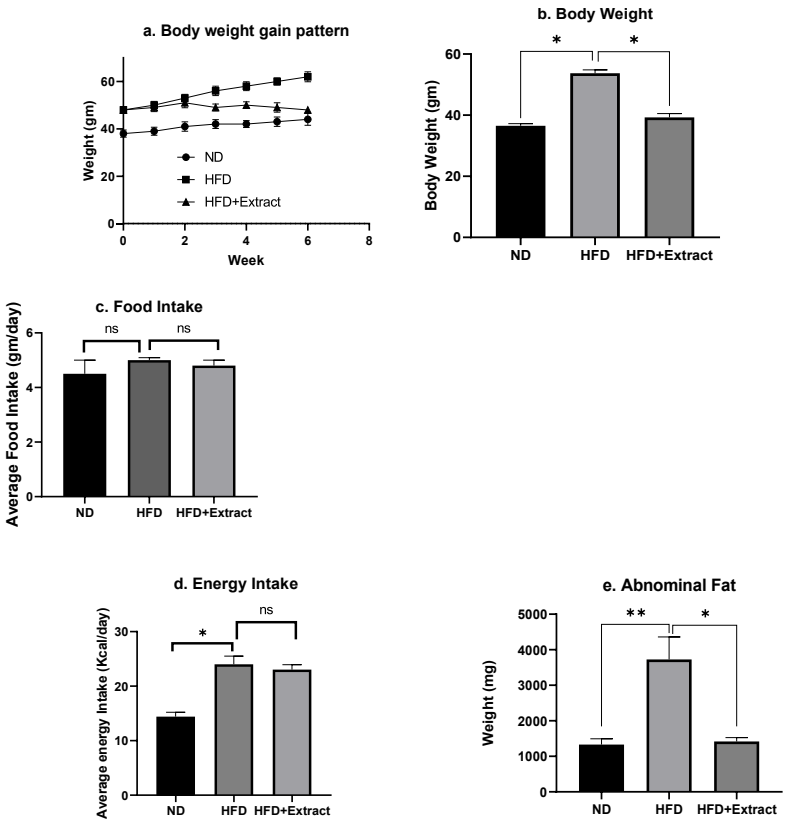


**Fig. 2.** Graphical presentation of (a) Body weight gain pattern, (b) Final body weight, (c) Average food intake. (d) Average energy intake, and (e) abdominal fat weight of three experimental mice groups. The values are presented as Mean ± SEM, significantly different from control at $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

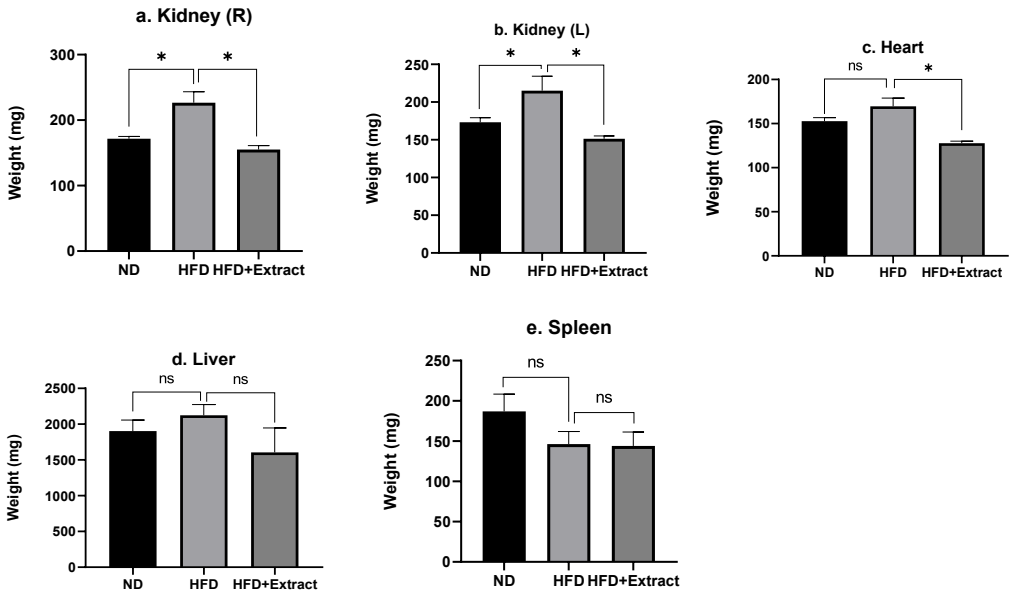


**Fig. 3.** Graphical presentation of organ weight. Kidney-R(a), Kidney-L (b), Heart (c), Liver (d) and Spleen (e) weight of three experimental mice groups. The values are presented as Mean ± SEM, significantly different from control at $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

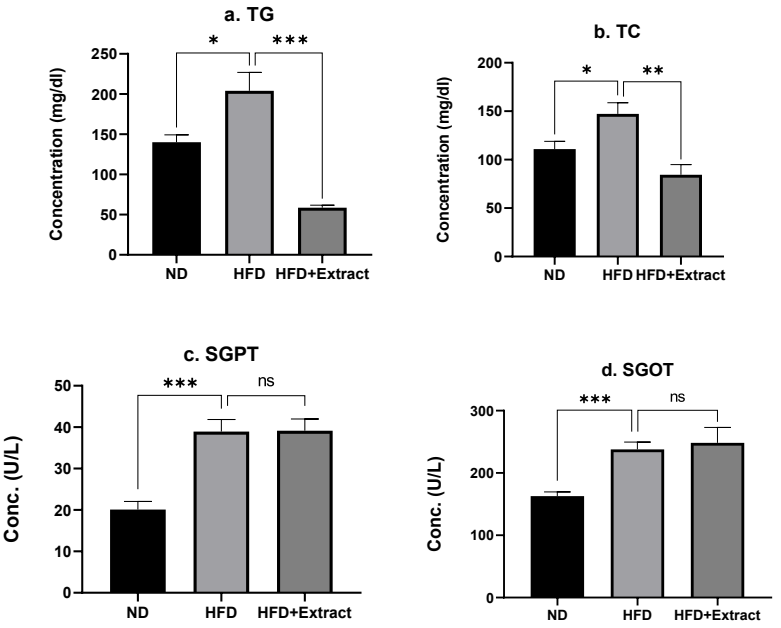


**Fig. 4.** Graphical presentation of lipid profile and liver function test; (a) triglyceride, (b) total cholesterol, (c) SGPT, and (d) SGOT of three experimental mice groups. The values are presented as Mean ± SEM, significantly different from control at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

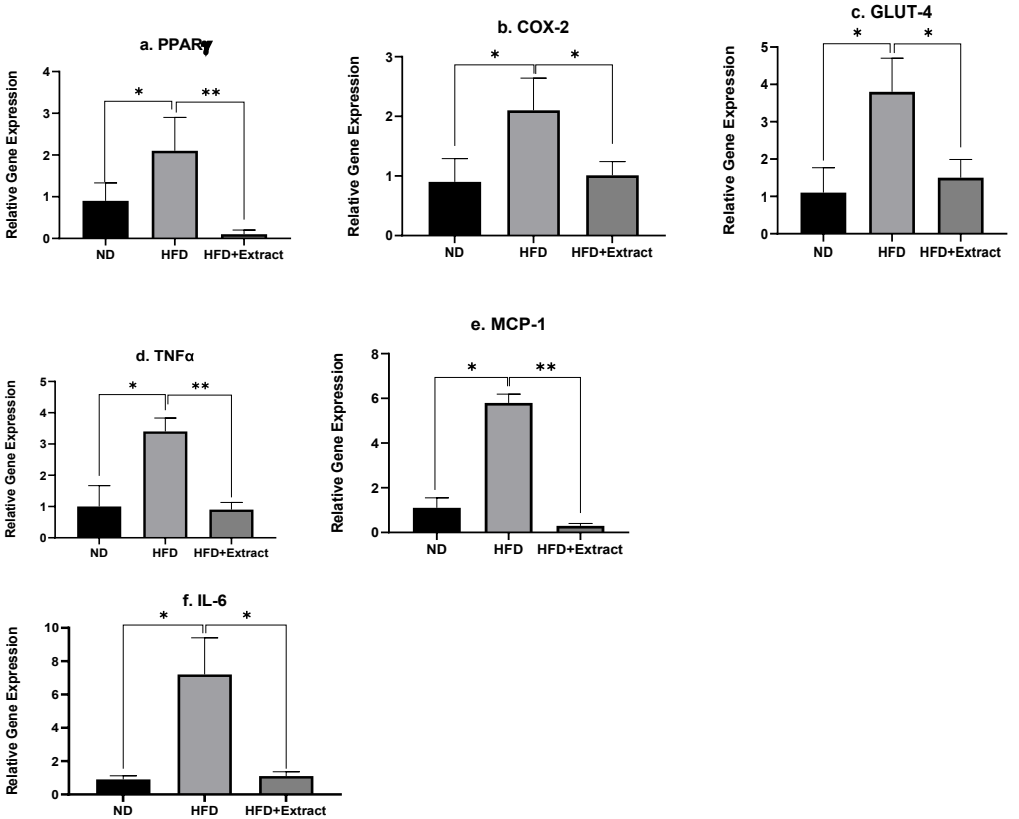


**Fig. 5.** Graphical presentation of gene expression of (a) PPARγ, (b) COX-2, (c) GLUT4, (d) TNFα, (e) MCP-1, and (f) IL-6 on experimental mice groups. The values are presented as Mean ± SEM, significantly different from control at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

## 4. Discussion

Obesity, a major public health issue, is considered a low-grade inflammation and has been identified as one of the primary risk factors for metabolic syndrome [15]. Thus, studying the pathophysiological process of adipogenesis and finding out the combating potential is gaining more interest nowadays.

Previously we documented *Musa accuminate,* a potential natural source for managing obesity and its complications via suppression of PPARγ in HFD-induced obese mice [8], in this study, we made use of *Alpinia calcarata* leaf extracts to demonstrate its anti-obesity and anti-inflammatory effects involving the attenuated expression of COX-2 and PPARγ in obese mice. Here we utilized high-fat diet-induced obese mice as it mimics most features of human physiology to develop obesity [16].

At first, we conducted an *in-silico* study to forecast the action of the plant *Alpinia calcarata* in reducing adipogenesis. The constituents found in *Alpinia calcarata* have the activity in reducing adipogenesis. Carotol, 1,8-cineole, camphor and methyl cinnamate have the activity of reducing adipogenesis by different pathways [12,17]. In our study, carotol have a highest binding affinity with PPARγ, and GLUT-4 and methyl cinnamate with COX-2. Carotol and β-pinene have the highest and same binding interaction with IL-6 among others. The most abundant amino acids are ILE42, ILE303, PHE307 and GLN299 are found in different compounds when binding with GLUT-4. VAL51 and MET357 are found in β-pinene and camphor when bound with IL-6 and PPARγ respectively. The ADMET analysis confirmed that the compounds have no toxic effect and they have good pharmacokinetic activity. After assuming it's activity then an *in-vivo* experiment was done in mice model.

To make the mice obese, we fed them a fat containing diet for a month. The establishment of obesity in mice was confirmed by the strikingly high weight gain and Lee index as reported in our other study [8,18]. High-fat diet (HFD) utilized here was able to induce obesity in our study mice as evidenced by a significantly increased the body weight as compared with the mice fed with a normal diet. The treatment with the methanol extract of *A. calcarata* was continued for the next six weeks to study its effect on obesity and its complications. In comparison to their obese counterparts alone, the extract-treated obese mice showed a significant ($p < 0.05$) reduction in body weight growth. The reduction in body weight was started just after the two week of treatment and continued throughout the study. Moreover, there were no significant differences in food intake behaviour among the groups of mice, but mice fed on HFD received significant higher amount of calorie compared with normal diet and the extract failed to have any affect. As the extract did not reduce the caloric intake but reduced the body weight in obese mice, this phenomenon indicates the capability of the extract to increase the energy expenditure to attenuate the body weight in obese mice. Neither we nor other group described the detail mechanism of energy expenditure process by *A. calcarata* leaf, this information will lead to explore the detail mechanism in future research. Additionally, in association with the trend of body weight pattern, HFD initiated the fat accumulation in the abdomen and the extract was effective enough ($p < 0.05$) to rescue the fat accumulation in HFD-induced obese mice. All these data explained the anti-obesity effect of our extract.

Furthermore, as the impact of obesity on particular tissues and systemic physiology to the extracellular and intracellular inflammatory signalling molecules has been described previously [19], we checked the changes in weight variation of different organs like the heart, kidney, and liver of our experimental mice. In this study, HFD tends to increase the weight of the organs studied here. Treatment with the extract showed the capacity to reverse the effect of HFD on the weight of the different organs. The heart and kidney weight have been reduced significantly, whereas changes in the liver and spleen weight were insignificantly. However, a report by

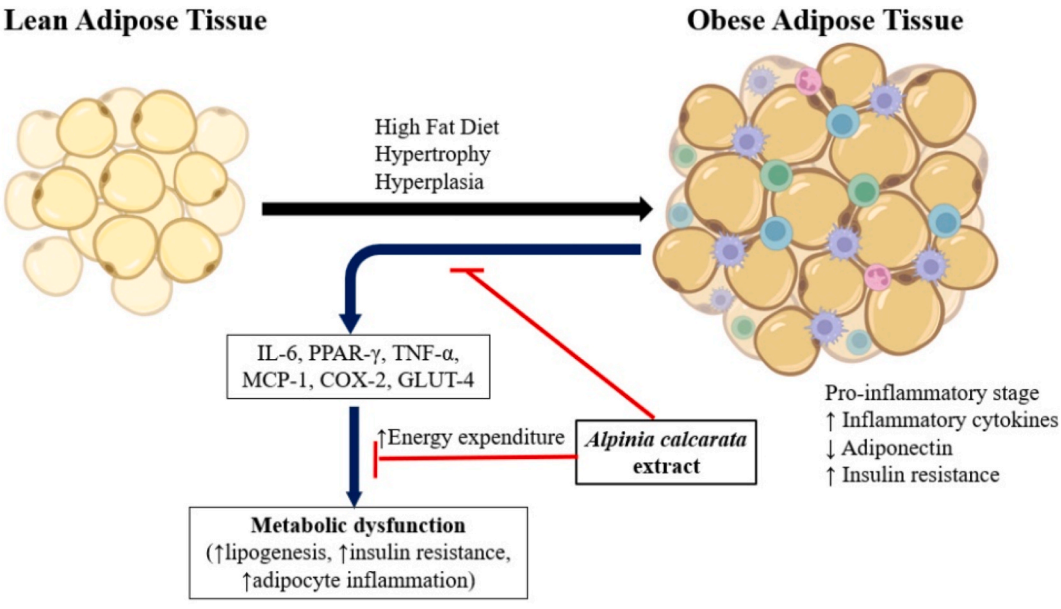


**Fig. 6.** Illustration of possible mechanism of action of *A. calcarata* leaf extract in modulating obesity and related complications.

Arambewela et al. [20] described no unexpected weight changes in different organs of normal rats treated with *A. calcarata* rhizome extract.

A significant elevation of serum lipids like total cholesterol, and total triglyceride is typically observed in obese animal, and people, and thus change in these lipid profiles are considered as an indicator of obesity. Our findings showed that, in comparison to the normal control, HFD considerably ($p < 0.05$) elevated lipid profiles, whereas treatment with *A. calcarata* extracts significantly ($p < 0.05$) decreased the serum lipid levels in HFD-induced obese mice. Furthermore, previously obesity was linked to non-alcoholic fatty liver disease and hence we sought to examine the hepatic enzyme levels as a function of hepatotoxicity. High-fat diet played a significant role in the elevation of SGPT and SGOT compare to that of control but the treatment with the extract did not show significant changes in serum hepatic enzymes levels indicating the action of *A. calcarata* is dependent on the pathophysiological conditions of the liver.

It is generally known that unless pancreatic lipase enzymes are active, dietary lipids in the gut are not absorbed. Polyphenols present in natural plant extracts have been studied for the inhibition of pancreatic lipase activity. Furthermore, natural polyphenols have been recorded to be linked with energy expenditure, suppression of appetite, and, adipocyte differentiation governing its role in obesity management [21]. *A. calcarata* leaf is a good source of polyphenols [22] that might alter the lipid metabolism or any other mechanism contributing to the suppression of HFD-induced obesity with attenuated risk of obesity-mediated metabolic complications. Moreover, very recently, carotol and 1,8 cineol are recorded to inhibit COX-2 [12,23]. COX-2 enzymes are responsible for the biosynthesis of pro-adipogenic prostanoids that activate the PPARγ and eventually lead to that accumulation in cultured matured adipocytes. In this study, we found elevated expression of COX-2 and PPARγ in HFD-induced obese mice but treatment with *A. calcarata,* rich in Carotol and 1,8 cineol [12], was sufficient to suppress mRNA expression of COX-2 and PPARγ in obese mice coupled with the decreased levels of triglyceride and cholesterol. Thus, manipulation in pro adipogenic prostaglandin synthesis by blocking the COX-2 pathway or direct suppression of PPARγ activity by *A. calcarata* plant extract contributed partly to reducing obesity in high-fat diet-induced obese mice. However, any additional mechanisms in reducing the adiposity by the tested extract cannot be overruled. Insulin-stimulated glucose uptake in adipocyte and skeletal muscles is regulated by GLUT4 under the control of PPARγ and is important to maintain glucose homeostasis. Over expressed GLUT4 increases the adipose tissue mass by increasing glucose transportation and altering the nutritional distribution in adipose tissue [24]. In our experimental settings, higher expression of adipose tissue-specific GLUT4 at the transcript level was observed in obese mice and the reduced expression was evidenced by the extract in comparison with the untreated obese group. However, the anti-hyperglycaemic activity of *Alpinia* spp. has been well documented [22]. The attenuated GLUT4 expression by *A. calcarata* leaf inhibit the glucose transportation in adipose tissue and eventually reduced the *de novo* fat synthesis in adipose tissue leading to attenuate the adipogenesis. This effect might be a feedback loop of GLUT4 in adipose tissue in maintaining the lipid haemostasis.

Obesity is regarded as a low-grade inflammatory condition and participates in the emergence of the metabolic syndrome. Adipose tissue-derived pro-inflammatory cytokines such as; TNFα, and IL-6 participate actively in the pathophysiology of metabolic syndrome. In addition, MCP-1 responsible for the migration of macrophages into the adipose tissue causes adipocyte dysfunction promoting adipocyte inflammation and metabolic syndrome [25]. Moreover, it has been established that the PPARγ-regulated expression of MCP-1 in mature adipocytes in culture is regulated by the COX pathway [26]. Here we tried to describe the role of *A. calcarata* to control the mRNA expression of adipose tissue-specific inflammatory cytokines like TNFα, IL-6, and MCP-1. As a result, in HFD-induced obese mice, we saw an increase in the transcript levels of these cytokines. In line with prior findings, our extract successfully reduced the expression of mRNA of these cytokines in obese mice, demonstrating the tested extract's anti-inflammatory properties. Natural sources have been well-documented for the control of obesity and obesity-mediated inflammation. Along with the ability to inhibit the COX-2, and LOX to exert the anti-inflammatory action of carotol, and 1,8 cineol [12,23], decreased the expression of TNFα, IL-6, and MCP-1 have been realized in different inflammatory conditions [27,28]. So, it is not unlikely that, the essential oils present in *A. Calcarata* leaf might evoke the anti-obesity and anti-inflammatory effect identically in HFD-induced obese mice.

Thus, taking all together, we are advocating that the bioactive components of *A. calcarata* leaf block the COX-pathway to reduce the action of PPARγ and ultimately reduce the action of TNFα, IL-6, and MCP-1 to exert anti-obesity and anti-inflammatory action in HFD-induced obese mice that is summarized in Fig. 6. Moreover, the isolation and identification of bioactive compounds and finding out the more detailed mechanism involved in this episode are under warrant.

## 5. Conclusion

Our data suggested that the methnol extract of *Alpinia calcarata* leaf can reduce obesity and obesity-mediated inflammation in HFD-induced obese mice coupled with the attenuated expression of COX-2, PPARγ, IL-6, MCP-1, TNFα, and GLUT-4. This finding endorses the utilization of *Alpinia calcarata* as a natural source of anti-obesity and anti-inflammatory agents.

## CRediT authorship contribution statement

**Imtiaz Ahmad:** Writing – original draft, Methodology, Investigation, Formal analysis. **Maruful Hasan:** Validation, Investigation, Conceptualization. **Dipty Rani Bhowmik:** Validation, Investigation, Conceptualization. **Rahima Begum:** Formal analysis, Data curation, Conceptualization. **Sourav Roy:** Visualization, Validation, Methodology, Conceptualization. **Md Monirul Islam:** Writing – original draft, Project administration, Conceptualization. **Md Abdur Rahman Ripon:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Mohammad Salim Hossain:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

## Informed consent statement

Not applicable.

## Institutional review board and ethics statement

The experimental protocol was approved by the Institutional Ethical Committee of Noakhali Science and Technology University (NSTU/SCI/EC/2022/106). All relevant rules, guidelines, and regulations were followed.

## Data availability statement

Some or all data, models, or code generated or used in the study are available from the corresponding author by request. The data are not publicly available due to privacy.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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