Cinnamomum verum-derived bioactives-functionalized gold nanoparticles for prevention of obesity through gut microbiota reshaping

Vivek K. Sharma¹, Prateeksha¹, Sateesh C. Gupta, Brahma N. Singh*, Chandana V. Rao, Saroj K. Barik**

Pharmacology Division, CSIR-National Botanical Research Institute, Lucknow, 226001, India

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ABSTRACT

Existing drugs have limited success in managing obesity in human due to their low efficacy and severe side-effects. Surface-modified gold nanoparticles have now received considerable attention of researchers for efficient biomedical applications owing to their superior uptake by cells, biocompatibility, hydrophilicity and non-immunogenicity. Here we prepared Cinnamomum verum derived bioactives-functionalized gold nanoparticles (Au@P-NPs) and assessed their impact on obesity and related immune-metabolic complications in high-fat diet (HFD)-induced obese mice using metabolic experiments along with 16S RNA gene-based gut microbial profiling and faecal microbiota transplantation (FMT). Au@P-NPs treatment prevented weight gain, decreased fat deposition, reduced metabolic inflammation and endotoxaemia in HFD-fed mice. Au@P-NPs-treated group exhibited better glucose tolerance and insulin sensitivity than HFD-fed control mice, and got completely protected against hepatic steatosis. These impacts were related to increased energy expenditure and enhanced Ucp1 expression in the brown adipose tissues of Au@P-NPs-administered animals, which strongly linked with the mRNA expression of the membrane bile acid receptor TGR5. Treatment of HFD-fed animals with Au@P-NPs altered plasma bile acid profile, and increased Akkermansia muciniphila and decreased Lactobacillus populations in the faeces. Au@P-NPs-treated animals revealed altered plasma bile acid profile, and increased Akkermansia muciniphila and decreased Lactobacillus populations in the faeces. FMT experiments showed lesser weight gain and greater energy expenditure in the mice fed with faecal suspension from Au@P-NPs-treated animals than that from HFD-fed mice. These results clearly establish that gold nanoparticles functionalized with bioactive compounds of C. verum have high potential to be an anti-obesity drug.

1. Introduction

Obesity along with related metabolic complications is now a major health concern worldwide and has become an economic and social burden [1]. It is predicted that at least one billion world population will be obese by 2030 [2]. Severe obesity contributes to the progression of a variety of disorders, including insulin resistance, type-2 diabetes, dyslipidemia, atherosclerosis and cancer [3,4]. Growing evidence has demonstrated that genetic and environmental factors are major causes of dysregulation of the balance between energy intake and expenditure which result in the storage of excess fat in adipose tissues [5]. Despite considerable efforts by the scientific community and pharmaceutical industry to discover new anti-obesity agents, obesity remains a major health challenge globally. The commercialized drugs viz., lorcaserin, topiramate and orlistat have very limited effect that reduce only 3–10% of the body weight. As such, most of them have been withdrawn from the market due to their severe side effects such as diarrhoea, headache, faecal incontinence and dyspepsia [6].

Recently, a growing body of research revealed a fundamental link between gut microbiota dysbiosis and obesity development, chiefly through regulation of nutrient acquisition, energy expenditure and fat deposition [7–11]. In most cases, obese people exhibit altered gut microbial diversity [12] and bacterial richness [9]. 16S rRNA gene-based sequence revealed a high ratio of the key phyla Firmicutes to Bacteroidetes (F/B), which could be linked with weight gain and metabolic dysfunctions [13]. Recent works have revealed that germ-free (GF) mice...
gain less body weight despite a high fat-diet intake than that of non-GF mice [14]. Moreover, after transplantation of gut microbiota in GF receivers using faecal re-suspension from donor obese mice, the obesity characteristics appeared in the receiver mice [15]. These accumulating evidences have suggested that gut microbiota play a major role in the regulation of obesity.

Bile acids are synthesized from cholesterol in the liver and are required for the absorption of lipids and fatsoluble vitamins [16]. Gut microbiota convert primary bile acids to secondary bile acids through various reactions, namely deconjugation, dehydrogenation, and dihydroylation [17]. In addition, bile acids stimulate Takeda G-protein coupled receptor 5 (TGR5) that enhance brown adipose tissue (BAT) activation, white adipose tissue (WAT) browning, and energy expenditure through UCPI signalling [18]. Thus, TGR5 and UCPI signalling modulation by bile acids through gut microbiota reshaping can provide a promising strategy for the prevention and treatment of obesity and related disorders.

Dietary polyphenols of *Cinnamomum verum* J. Presl (family: Lauraceae) have the potential to reduce weight gain, inflammation and insulin resistance, producing potent anti-obesity effects via gut microbiota remodeling [19–21]. However, these effects proven through preclinical and clinical studies are inadequate because of the limited bioavailability of therapeutically active phytochemicals of *C. verum* in gut environment [22]. *Ayurveda*, an ancient Indian medicine system that dates back to 5000 B.C., uses phytochemicals-coated gold nanoparticles (AuNPs) also known as *Swarna bhasma* for enhancing bioavailability of phytochemicals to treat bronchial asthma, rheumatoid arthritis, diabetes mellitus, liver, and indigestion [21,23]. A similar approach has also been followed by Chinese and Egyptian civilization since 2500 B.C [23,24]. The surface-functionalized NPs, particularly AuNPs are increasingly being used for drug delivery and diverse biomedical applications in recent years due to their excellent bio-compatibility, hydrophilicity, non-immunogenicity properties, and superior therapeutic benefits [25–34]. Plant metabolites-coated AuNPs could reduce fat accumulation in 3T3-L1 adipocyte cells through adipogenesis inhibition, an essential process responsible for forming fat cells [35]. On the other hand, the underlying mechanisms of these effects of AuNPs have not yet been fully elucidated. Earlier studies have demonstrated the role of various plant polyphenols in BAT activation through gut microbiota reshaping that leads to enhanced WAT browning and energy expenditure [36–41]. This encouraged us to study if surface-functionalized AuNPs exert anti-obesity effect via gut microbiota remodeling. Thus, the objective of this investigation was to develop *C. verum* derived bioactives-functionalized Au-NPs (Au@P-NPs) and elucidate their mechanisms underlying the beneficial effects on diet-induced obesity and related immunometabolic complications. The results of our investigation clearly reveal the possible mechanism of actions by which biocompatible Au@P-NPs increase BAT activation and WAT browning and might offer Au@P-NP as an anti-obesity drug.

2. Materials and methods

2.1. Materials

We procured chloroauric acid (HAuCl₄) and sodium citrate from HiMedia Laboratories Pvt. Ltd., (Maharashtra, India). We bought polyvinylpropylene from Sigma-Aldrich, USA. *C. verum* bark was procured from a local market. We received ELISA kits of IL-1β, IL-6, INF-γ, VEGF, and RANTES from R & D Systems, Canada. CD36, FAS, FABP4, PPARγ, and ACC antibodies were procured from Thermo Fisher Scientific and Abcam.

2.2. Synthesis and characterization of NPs

We prepared citrate-capped gold nanoparticles (Au-NPs) and *C. verum* derived bioactives-functionalized Au-NPs (Au@P-NPs) in our laboratory. The detailed methods of their synthesis has been provided in the Supplementary Information section. We characterized the synthesized NPs by UV–vis spectrophotometer (UV201, Thermo Fisher Scientific, USA). A drop of diluted NPs was deposited on a copper grid and left to dry for 12 h at room temperature. We observed the NPs with a transmission electron microscope (TEM) at 200 kV (JEOL JEM-2100, Japan). The average sizes and zeta potentials of NPs were measured by a Zetasizer MAL101294 (Malvern, UK). Fourier-transmission infrared spectroscopy (FTIR) was used to examine the functionalization of phytochemicals of *C. verum* on the surface of NPs (PerkinElmer Life and Analytical Sciences, CT, USA).

2.3. Animals

Eight-weeks-old male Swiss mice were obtained from CSIR-Central Drug Research Institute, India and independently housed in a controlled room (22 ± 1 °C temperature; 45–55% relative humidity) under 12 h daylight and 12 h without light with free access to sterile food pellets and water ad libitum in the animal house. The experiments were performed in accordance with the guidelines of the Committee for the Purpose of Supervision on Experiments on Animals (CPCSEA), India, as approved and promoted by the Institutional Animal Ethics Committee (Registration No. 1732/GO/Re/S/13/CPCSEA). After 2 weeks of acclimatization on a chow diet (CD: Bajaj Instrument Works, Ghaziabad), animals were arbitrarily distributed into 4 groups (n = 6) and fed on CD or a high-fat diet (HFD) (Table S1). Treatment initiated along with the beginning of HFD and contained every day to two oral doses (10 mg/kg) of resuspended Au-NPs and Au@P-NPs in drinking H₂O of animal facility throughout 8 weeks. The vehicle-treated (H₂O) groups are referred to as CD and HFD. Body weight increase and food eating were measured two times in a week. At week 8, mice were anesthetized by CO₂ asphyxiation and subsequently sacrificed prior to awakening from anesthesia. Blood was collected by cardiac puncture in heparinized (2 IU) capillary tubes, and without delay centrifuged at 3000 rpm for 30 min at 4 °C to separate plasma from cells. Plasma layer was kept at −80 °C until processing. Fat pads, namely brown, subcutaneous and visceral were cautiously abstracted together with gastrocnemius muscle, liver, lungs, kidneys, heart and pancreas (Fig. S1). The detailed methods and analyses applied to examine the gut microbial taxonomic abundances in mouse faecal samples are provided in the Supporting Information.

2.4. Total RNA extraction and qPCR analysis

We homogenized freeze-dried powdered samples of collected tissues in1 ml of QiAzo1 lysis reagent (Qiagen, Germany). Total RNA was extracted and purified using an RNeasy mini kit (Qiagen, Germany). cDNA was constructed with total RNA (1 μg) using the Verso cDNA synthesis kit (Thermo Fisher Scientific, India) following the manufacturer’s instructions. The qPCR was carried out using SYBR Green master mix (Thermo Fisher Scientific, India) with 1:10 diluted cDNA on a 7900HT real-time system (Applied Biosystems, USA). The relative expression of genes of interest was assessed by the ΔΔCt method. The primers used to detect genes are structured using the program, Primer3 v.0.4.0, and gene sequences are mentioned in Table S2.

2.5. Glucose homeostasis

For insulin (INS) tolerance tests, we starved mice for 6 h (8 a.m. To 2 p.m.) at week 7. We administered INS with a dose of 1 IU/kg through i.p. injection. We measured glycaemia (blood glucose content) by a glucometer (Roche, Germany) using the blood obtained from the tail before INS administration, and at 10, 20, 30, and 60 min after INS administration. For oral glucose tolerance test (OGTT), animals were starved overnight (8 p.m.–8 a.m.) at the end of week 6 and glucose at the dose of 1 g/kg body weight was given orally. We quantified glycaemia with a glucometer using the blood collected from the tail prior to glucose administration and at 0, 15, 30, 60, 90 and 120 min following glucose
administration. Approximately 30 μL of blood was used during OGTT for insulinemia measurement. We measured the homeostatic model assessment of insulin resistance (HOMA-IR) index using the formula of Anhe et al. [3].

2.6. Faecal microbiota transplantation (FMT) experiments

FMT experiments were carried out with faecal resuspension (FRS) from mice fed HFD plus Au@P-NPs for 8-weeks. The harvested faecal pellets (110 mg) were transferred to anaerobic chambers for making suspension in 1x PBS (350 μL) [3]. Eight-weeks-old 14 GF mice were maintained under aseptic conditions, and animals were treated forcefully with immediately made 350 μL of FRS orally. Germ-free (GF) mice re inoculated with the faecal microbiota of HFD-fed control animals (denoted as HFD-recipients; n = 5) and Au@P-NPs-treated (denoted as Au@P-NPs recipients; n = 5). Recipient mice were first kept in metabolic cages (Sable International Systems, USA) for 4 days and then shifted to ventilated GF cages for next 10 days and maintained on less-fat (10%) containing diet during the early days of post-colonisation (PC) (Fig. S2).

The protocols applied to examine bacterial quantification by qPCR, metabolic rate, interscapular temperature, plasma bile acids, protein expression, H&E staining, primer sequences, and composition of diets are available at the online Supporting Information.

2.7. Statistical analysis

All values are presented as mean ± standard error (SE). We used one-way ANOVA with Bonferroni post hoc test (BPHT) and Kruskal-Wallis test (KWT) with Dunn’s multiple comparison tests (DMCT) for parametric and non-parametric results, respectively to allocate significance to the differences among groups (SPSS 13.0, USA). We used two-way ANOVA with a BPHT (Sigmaplot, USA) when time was considered as a variable.

3. Results and discussion

We examined the morphology and size of Au-NPs and Au@P-NPs by TEM. The NPs distributed uniformly, and the average diameters of Au-NPs and Au@P-NPs were approximately 17 nm (Fig. 1a–d). The hydrodynamic diameters of Au-NPs and Au@P-NPs were 27.6 and 29.1 nm, respectively and the corresponding zeta-potential values were –12.4 mV for Au-NPs and –15.6 mV for Au@P-NPs (Table S3). The polydispersity index (PDI) of Au-NPs was 0.278, while Au@P-NPs revealed PDI of 0.286, indicating the monodispersity of Au@P-NPs. To determine the stability of synthesized NPs, PDI values were measured after twelve months of storage at 25 ± 1 °C. Au@P-NPs revealed excellent mono-dispersity (PDI-0.293), while PDI of Au-NPs increased from 0.278 to 0.312. We identified 13 metabolites, viz., 3-hydroxybutyrate, cinnamyl acetate, glucose, myoinositol, glycerol, galactose, fumaric acid, catechin, rutin, pyrogallol, p-coumaric acid, cinnamic acid and cinnamaldehyde which were involved in the coating of NPs (Fig. 2; Table 1). Although galactose is the major component of Au@P-NPs, several studies have reported that galactose and its derivatives act as linker agents to assemble various functional agents on the surface of metal NPs [48,49]. As such, the content of galactose present on the surface of Au@P-NPs was so less that it was unlikely to cause hyperglycemic-mediated obesity [50] rather it might have favoured gut microbes responsible for maintaining gut-microbiota-bile acid axis. In addition, p-coumaric acid [38], pyrogallol [51], cinnamaldehyde [52], fumaric acid [53] and cinnamic acid [54,55] present on the surface of Au@P-NPs have been reported for inducing thermogenesis and brown adipose tissue (BAT) activation in adipose tissues (ATs) to provide anti-obesity effects. Similarly, other cinnamyl acetate, rutin and catechin are known for anti-inflammatory activity [16,56,57] which can regulate metabolic inflammation in ATs of HFD-fed mice.

We administered Au-NPs and Au@P-NPs orally once a day to the obese mice induced by HFD throughout 8-weeks at a concentration of 10 mg/kg to assess their beneficial effect on obesity and related metabolic disorders. Au@P-NPs treatment prevented gain in HFD-induced body weight from day 14 onwards (Fig. 3a and b) and these results were not related to alterations in energy intake (Fig. 3c). The accumulation of fat in ATs is often related to obesity and related disorders [3,36]. Therefore, we examined whether the Au@P-NPs-induced prevention of weight gain is associated with alterations in ATs. Au@P-NPs prevented fat accumulation in all ATs namely, epididymal white AT (eWAT), retroperitoneal white AT (rWAT), mesenteric white AT (mWAT), inguinal white AT (iWAT) and interscapular brown AT (iBAT) (Fig. 3d). Treatment of Au@P-NPs did not affect the weight of heart, gastrocnemius and pancreas as well as length of intestine, and caecum quantity (Fig. S4a–d). When compared with the CD group, liver weight in HFD-fed group significantly raised, but HFD-persuaded liver weight and hyperlipidemia were markedly prevented by Au@P-NPs administration (Fig. 3e), as revealed by reduced contents of triglyceride, total cholesterol and LDL-c in both liver homogenate (Fig. 3f) and plasma (Fig. 3g) of Au@P-NPs group against HFD control group, measured by colorimetric kits. We noticed non-significant difference in everyday faecal energy production between Au@P-NPs-treated and HFD control groups, indicating that Au@P-NPs did not modify food assimilation (Fig. 3h). Au@P-NPs treatment enhanced energy consumption, which was not related to reinforce physical movement (Fig. 3i; Fig. S4g–i), ensuring a smaller amount of energy available for deposition (Fig. 3j). In line with elevated oxygen utilization, Au@P-NPs-treated animals revealed an increasing interscapular temperature (36.66 °C ± 0.07) than HFD-fed (35.69 °C ± 0.08) animals (Fig. 3k). On the other hand, Au-NPs did not impede HFD-induced weight gain and fatty liver parameters. Au@P-NPs were much more efficient in the prevention of weight gain, adiposity and dyslipidemia by accelerating energy consumption and thermogenesis than Au-NPs. These findings indicate that the involvement of anti-obesity phytochemicals of C. verum in coating of the NPs which play a key role in preventing the weight gain and inducing thermogenesis by Au@P-NPs in HFD-induced obese mice.

We next studied the levels of interleukin-1 beta (IL-1β), IL-6 and interferon-γ (INF-γ) in serum using ELISA kits to assess metabolic inflammation in the AT of HFD-fed mice without and with treatments. The mice belonging to HFD control and Au-NPs-treated groups exhibited greater IL-1β, IL-6 and INF-γ levels than the Au@P-NPs group (Fig. 4a–c). We observed reduced levels of C–C chemokine ligand 2 (RANTES, a chemokine, VEGF (vascular endothelial growth factor), and chemokine ligand 2 (CCL2/MCP-1) in the Au@P-NPs group compared to the HFD control group (Fig. 4d–f). Corresponding to decreased CCL2, an essential regulator of macrophage infiltration in the AT, we observed reduced expression of Adgre (F4/80) mRNA (Fig. 4g). Au@P-NPs revealed decreased adipocyte size (Fig. S5a–c) and it has been reported that large adipose cells trigger an inflammatory cascade in ATs [58]. Au@P-NPs
administration also obviated metabolic endotoxaemia, as shown by reduced LPS concentration in mice bloodstream (Fig. 4h), suggesting regulatory effect of Au@P-NPs on AT and circulatory cytokines. While fasting blood glucose did not change significantly between Au-NPs and HFD control groups (Fig. 4i), Au@P-NPs treatment did obviate fasting hyperinsulinemia (Fig. 4j), resulting in the enhanced fasting insulin sensitivity (IS) as revealed by reduced HOMA-IR, a technique applied to measure β-cell function and insulin resistance (Fig. 4k). OGTT revealed that Au@P-NPs improved glucose tolerance (Fig. 4l and m) and restricted HFD-induced hyperinsulinemia (Fig. 4n and o). The IS 10 min after subcutaneous injection of insulin got improved in Au@P-NPs group as compared to HFD control group (Fig. 4p). In summary, these results suggest that Au@P-NPs treatment improves glucose homeostasis and IS by preventing the HFD-induced obesity and visceral fat deposition and inflammation. Since obesity is the results of high fat/or sucrose induced imbalance...
in gut microbial community, remodeling of gut microbial diversity is an important strategy in the prevention of obesity [3,59]. Mounting evidences have associated with the reduction of beneficial gut microbiota in obese people [8,10,11]. To confirm the consistency of the microbial profile, faecal specimens were collected at the end of the study and at the beginning of the study after acclimatization of mice on a CD. We sequenced 16S rRNA genes by Illumina MiSeq platform, and assessed the gut microbial profile as sequenced, V3–V4 region of 16S rRNA genes using a 2 × 300PE v3 sequencing kit. Principal coordinate analysis (PCoA) showed that the traditional method of CD-fed group clustered at baseline, 8 weeks of HFD feeding severely altered the intestinal microbial profile (Fig. 5a). Au@P-NPs-administered group, however, clustered partly at a distance of HFD control group specimens, signifying fundamental alterations in Au@P-NPs intestinal microbial profile (Fig. 5a). Au@P-NPs administration obviated the HFD-induced reduction in gut microbial richness, as indicated by Chao1 index (Fig. 4b) and F/B ratio (Fig. 5e and d), two key indicators of gut microbial diversity [9,60]. At genus level, Au@P-NPs protected the HFD-induced reduction in Akkermanis(s, Bifidobacterium, Barnesiella and Proteobacteria, and decreased the relative abundance of Lactobacillus, Streptococcus, Acinetobacter and Papillibacter (Fig. 5e). We observed the most of the decline in Firmicutes in the gut microflora of Au@P-NPs-treated group as compared to HFD control group (Fig. 5d).

The linear discriminant analysis effect size (LEfSe, an algorithm used for biomarker discovery and plotting in microbiome studies) method ranked Bacteroides, Parabacteroides and Barnesiella coupled with a foremost reduction in operational taxonomic units (OTUs, applied to categorize groups of closely related individuals) designated to Lactobacillus spp. and Streptococcus spp. as the major attributes distinguishing faecal microbial populations of Au@P-NPs group from that of HFD control group (Fig. 5h). As we noticed a propensity towards increase of Akkermanis muciniphila and Bifidobacterium in Au@P-NPs group (Fig. 5e), we quantified these bacteria by qPCR technique and detected a foremost enhance in their population in Au@P-NPs group (Fig. 5i and j). Increasing abundances of A. muciniphila and Bifidobacterium were reported demonstrating the metabolic benefits of these gut bacteria [61, 62]. Several researchers have earlier confirmed the relationship between consumption of dietary polyphenol-rich extracts and enhanced abundance of Bifidobacterium and A. muciniphila [63]. Our study also revealed the presence of polyphenolic compounds of C. verum on the surface of Au@P-NPs, which have known positive effects on A. muciniphila. Polyphenolic compounds found in the cell wall of certain plants increase the abundance of A. muciniphila and other probiotic bacteria [3,64,65]. Au@P-NPs is also coated with various polyphenolic compounds, such as cinnamic acid, cinnamaldehyde, ferulic acid, rutin, and catechin seem to promote the growth of beneficial gut bacteria, particularly A. muciniphila and Bifidobacterium in vivo. In early weaned rats, cinnamic acid and cinnamaldehyde from C. verum have been reported to decrease cardiometabolic risk through glucose uptake inhibition and appetite modulation by remodeling gut microbiome [66,67]. Similarly, rutin, another polyphenolic compound was reported to attenuate gut dysbiosis and inflammation in paneth cells of obese mice [68]. The positive effect of catechin, another dietary flavonoid present in green tea and C. verum, on gut microbes is also well-established in several pre-clinical studies [69, 70]. Thus, the presence of above ingredients of C. verum on the surface of NPs with proven gut microbial regulatory effects provides an explanation how Au@P-NPs modulates gut microbiota of HFD-fed mice.

While OTUs designated to the genera Bacteroides and Parabacteroides (Fig. 5h), their abundances were considerably low (Fig. 5e). We thus decided to focus on the significant Au@P-NPs-associated actions, particularly on the phytotypes such as Lactobacillus, Streptococcus, Acinetobacter, A. muciniphila, Barnesiella and Bifidobacterium. Besides, no noticeable histological injury in major organs such as liver, lungs, kidney, heart and pancreas (Fig. 6) and no significant alteration in the level of blood serum parameters (Table 2) were observed in 8-weeks-old mice treated with Au@P-NPs as compared to CD-fed group, demonstrating the biocompatibility of the Au@P-NPs.

As bile acids are known to regulate glucose, lipid and energy metabolism in BAT and skeletal muscle [18,71], we wanted to assess whether alterations in the bile acids profile can be related to the impacts observed in Au@P-NPs-treated group. Although the relative proportion of

| Table 1 |
| --- |
| Chemical shift | Multiplicity | Metabolite | Content (μg/mg Au@P-NPs) |
| d | 3- | Hydroxybutyrate | 1.81 ± 0.11 |
| s | Cinnamyl acetate | 0.66 ± 0.35 |
| t | Glucose | 2.44 ± 0.15 |
| dd | Myoinositol | 4.48 ± 0.27 |
| m | Glycerol | 3.75 ± 0.24 |
| dd | Galactose | 17.73 ± 1.16 |
| s | Ferulic acid | 1.14 ± 0.11 |
| d | Catechin | 0.43 ± 0.02 |
| d | Rutin | 2.11 ± 0.16 |
| m | Pyrogallol | 0.96 ± 0.04 |
| m | p-Coumaric acid | 6.04 ± 0.31 |
| m | Cinnamic acid | 1.46 ± 0.13 |
| d | Cinnamaldehyde | 2.68 ± 0.16 |

Results are presented as the mean ± SE.
ω-muricholic acid (ω-MCA; a secondary bile acid) was higher in the plasma of Au@P-NPs-treated group than the HFD-fed group, the abundance of conjugated bile acids such as tauro-α-murocholic acid (TαMCA) and tauro-β-murocholic acid (TβMCA) were lesser in the former group (Fig. 7a and b). Besides, the relative proportions of unconjugated bile acids, viz., CDCA (chenodeoxycholic acid), DCA (deoxycholic acid), hyodeoxycholic acid and UDCA (ursodeoxycholic acid) were greater in the plasma of Au@P-NPs-treated group than the HFD-fed control group (Fig. 7b; Fig. S6a-h). Bile acid content in faecal material was similar in HFD-fed control and Au@P-NPs-treated groups (Fig. 7b; Fig. S6a-h), indicating that changed circulatory bile acids in Au@P-NPs group was related to variation in bile acid production rather than excretion/re-uptake. Based on the obtained results, we cerebrated that farnesoid X receptor (FXR)-stimulating bile acid synthesis may...
Fig. 4. Treatment of Au@P-NPs and Au-NPs on inflammation and glucose homeostasis in HFD-fed mice. Protein lysates of eWAT of mice were used to measure (a) IL-1β, (b) IL-6, (c) INF-γ, (d) RANTES, (e) VEGF and (f) MCP-1. (g) Relative mRNA expression of Adgre in eWAT by qPCR. (h) LPS content in blood plasma of 6 h starved animals. Animals were starved from 6 p.m. to 6 a.m. (12 h) at week 6 and subjected to OGTT. NPs were orally given 120 min before to the experiment, and the status of glycaemia was measured 15 min after NPs treatment to examine potential alterations in glycaemia related with sugars present in Au@P-NPs to observe likely alterations in glycaemia linked with sugars of C. verum present in AECV. (i) Glycaemia (fasting) index. (j) Insulinaemia (fasting) index. (k) HOMA-IR. (l) OGTT/glycaemia (nM). (m) OGTT in terms of an area under the curve (AUC). Blood samples were collected during OGTT and employed after glucose administration to measure (n) Insulinaemia during OGTT and (o) Insulinaemia in terms of AUC. Animals were starved for 6 h at week 7 and (p) ITT (insulin tolerance test) was performed after insulin injection at the dose of 1.0 IU/kg through i.p. route. (n, o and p) Two-way frequent measurements ANOVA with BPHT. (a–k, m and o) One-way frequent measurements ANOVA with BPHT. *P < 0.05, **P < 0.01 and ***P < 0.001 for CD against HFD; $P < 0.05, $$P < 0.01 and $$$P < 0.001 for Au@P-NPs against HFD.
Fig. 5. Effect of Au@P-NPs on obesity-associated gut microbiota imbalance in HFD-fed mice. At week 8, faecal pellets of indicated treatments were collected. Faecal samples were also harvested during the last week of adaptation on a CD (indicated by bl), before the administration of the HFD or Au@P-NPs. After getting good quality of genomic DNA, 16 S rRNA gene-based gut microbiota profiling was carried out. (a) PCoA. (b) Chao1 index at OTU level. (c) F/B ratio. The relative richness of phylotypes at (d) phylum and (e) lowest taxonomic level (LTL) attained. LEfSe was figured out to investigate the taxa within the LTL likely that more sturdily distinguish between the gut microbiota of (f) CD vs HFD, (g) Au-NPs vs HFD, and (h) Au@P-NPs vs HFD. (i) qPCR amplification of A. muciniphila normalized by 3 × 10^9 copies of 16 S (total bacteria) in faecal sample. (b–e) KWT with DMCT. (i) One-way frequent measurements ANOVA with a BPHT. *P < 0.05, **P < 0.01 and ***P < 0.001 for CD vs HFD; $P < 0.05, $$P < 0.01 and $$$P < 0.001 for Au@P-NPs vs HFD.
recompense for the prohibitory action of decreased bile acid pool size, thus hampering the FXR-led negative reaction link regulatory bile acid profile in Au@P-NPs-treated group and retaining the contents of plasma bile acids lower in comparison to HFD-fed control group. While more studies in the future are required to fully decode the mode of actions by which Au@P-NPs affect bile acid equilibrium, it is of primary concern that such FXR-activatory bile acid pool may repay for the impending involvement of changed bile acid profile (Fig. 7f and g). Further supporting iWAT browning, Au@P-NPs group revealed a trend of upregulated mRNA expression of Prdm16 (PR domain containing 16) and Ucp1 in iWAT compared to the HFD-fed control group (Fig. 7f). With enhanced BAT activity, Au@P-NPs increased the mRNA expression of Prdm16 and Pgc1a (PPARγ coactivator 1α) in the BAT as compared to HFD-fed control mice (Fig. 7g). The Fgf15, an intestinal hormone has been reported to activate the process of thermogenesis and browning of WAT by inducing Fgfr4 activation [77]. Consequently, we noticed higher Fgfr4 mRNA expression in the iWAT than in the eWAT and BAT of Au@P-NPs-treated group (Fig. 7e-g), suggesting that increased browning of subcutaneous white fat in this group is linked to enhanced sensitivity to Fgf15. We observed a strong correlation between the mRNA expression profiles of many genes implicated in activation of BAT and iWAT browning (Fig. 7h). Ucp1 and Dio2 genes in BAT had strong correlation with Ucp1 and Dio2 in iWAT. In addition, within the BAT, TGR5 was strongly correlated with Pgc1a, and within the iWAT, Ucp1 and Dio2 were correlated with Dio2 and fgfr4, respectively. Watanabe et al. in their study concluded that A. muciniphila is positively correlated with plasma CDCA and particularly the secondary bile acids, viz., DCA and UDCA that worked via TGR5 to increase the brown lipocytes and non-shivering thermogenesis (heat generation owing to metabolic energy alteration by burning of WAT) by promoting intracellular thyroid hormone activation [18]. Thus, the circulating bile acids levels observed in our study in Au@P-NPs-treated animals were related to the increased BAT activation and browning of subcutaneous WAT through enhanced energy expenditure which might have been modulated through the gut microbiota.

However, quantifying how dietary polyphenolic compounds alter the intestinal microbial atmosphere to support the growth of A. muciniphila needs additional study. The interaction between this bacterium and bile acid pool size also needs to be investigated. No pathway connection to deconjugation/dehydroxylation of bile acid in the genome database (KEGG) of A. muciniphila was found. The capability of A. muciniphila to transform and resist to the antimicrobial action of various bile acids though indicated, further lab-based validations are needed. Although the direct involvement of changed bile acid profile size and composition observed in Au@P-NPs-treated group in increasing energy expenditure needs further study, the present study did bring novel insights into the cross-talk between bile acid and gut microbiota, and its association with the anti-obesity effects of phytochemicals-functionalized AuNPs.

We also assessed whether alterations in lipolytic/lipogenic event also contribute to decreased fat accumulation observed in Au@P-NPs-treated
Table 2

| Parameter | CD | Au@P-NPs |
|-----------|----|----------|
| **Blood biomarkers:** | | |
| RBCs (10^12/L) | 4.83 ± 0.29 | 5.14 ± 0.028 |
| WBCs (10^9/L) | 7.37 ± 0.55 | 7.88 ± 0.62 |
| HGB (g/dL) | 14.4 ± 1.13 | 14.1 ± 1.04 |
| CRP (mg/L) | 0.97 ± 0.052 | 1.03 ± 0.021 |
| TG (mg/dL) | 101.2 ± 8.44 | 104.1 ± 8.28 |
| **Liver biomarkers:** | | |
| ALT (U/L) | 82.2 ± 7.74 | 87.1 ± 8.52 |
| AST (U/L) | 124.4 ± 11.5 | 131.4 ± 14.1 |
| ALP (U/L) | 29.7 ± 1.93 | 28.7 ± 1.37 |
| TB (mg/dL) | 0.69 ± 0.041 | 0.72 ± 0.041 |
| **Kidney biomarkers:** | | |
| Creatinine (mg/dL) | 1.54 ± 0.14 | 1.51 ± 0.013 |
| BUN (mg/dL) | 17.9 ± 1.38 | 18.9 ± 1.72 |
| **Lipid biomarkers:** | | |
| HDL-c (mg/dL) | 81.9 ± 8.16 | 85.1 ± 7.61 |
| TG (mg/dL) | 42.4 ± 4.16 | 43.7 ± 5.1 |
| LDL (mg/dL) | 93.6 ± 7.51 | 92.8 ± 9.72 |

Results are presented as the mean ± SE.

RBCs, red blood cells; WBCs, white blood cells; HGB, haemoglobin; CRP, C-reactive protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TB, total bilirubin; BUN, blood urea; TC, total cholesterol; HDL-c, high density lipoprotein cholesterol; TG, triglyceride; BUN, blood glucose.

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The expression of lipoprotein lipase (LPL) inhibitor fasting-induced adiponectin (Fiaf) and cleavage stimulated by fatty-acid synthase (Fas) in ileum of A. muciniphila increased by Au@P-NPs-treated group in faecal samples of HFD-receiver animals. The remains faecal suspensions from Au@P-NPs-treated animals on day 2 PC were mainly led by a greater population of Verrucomicrobia phylum exclusively represented by A. muciniphila in the gut microbiota of rodents and humans [80]. The abundance of Faecalibacterium prausnitzii got elevated in Au@P-NPs-receiver group on days 1 and 2 PC (Fig. 8d and e). On day 2, Au@P-NPs recipients exhibited higher abundances of A. muciniphila and Bifidobacterium and lower proportions of Lactobacillus and Acinetobacter (Fig. 8e and f). On day 3 PC, enhanced supremacy of Bifidobacterium and Barnesiella on days 1 and 2 PC, lower Streptococcus on day 1 PC and higher A. muciniphila on day 2 PC, characters noticed in Au@P-NPs-receiver and also seen in Au@P-NPs-administered donor groups. The results recommend that A. muciniphila got elevated in Au@P-NPs-receiver group in faecal suspensions of Au@P-NPs-administered group faecal microbial communities were adequate to enhance energy expenditure in the used mouse model. FMT experiments also showed that the gut microbiota of Au@P-NPs-administered animals could moderately colonize in GF recipient animals. We performed qPCR to examine the proportion of foremost phyotypes increased by Au@P-NPs-treated group in faecal samples of HFD receivers. The lower abundance of Streptococcus spp and enhanced proportion of A. muciniphila and Bifidobacterium were noticed in the faecal samples of Au@P-NPs receivers as compared to HFD receivers on day 1 PC (Fig. 8h–j). Interestingly, both A. muciniphila and Bifidobacterium stayed considerably abundant in the faeces of Au@P-NPs-receiver animals as compared to HFD receivers on days 2 and 3 PC (Fig. 8i and j). The abundance of Barnesiella was found higher on day 2 PC in Au@P-NPs donors (Fig. 8k). The abundance of Lactobacillus was noticed lower on day 2 PC in Au@P-NPs-receiver group (Fig. 8l). Similarly the abundance of Acinetobacter and Parabacteroides was found lower on day 2 PC in Au@P-NPs receivers in comparison to HFD-receivers (Fig. 8m and n). These results indicate that A. muciniphila and Bifidobacterium are main microflora for enhanced energy expenditure and decreased weight gain on Au@P-NPs treatment in HFD-induced obese mice. This is backed by several earlier investigations revealing that the oral transplantation of A. muciniphila to HFD-fed animals induced weight gain and related metabolic disorders [61,79,81,82]. But the population size of A. muciniphila got elevated at the 3 day PC, as oxygen utilization and body mass alteration become similar between Au@P-NPs-receiver and HFD-receiver animals, indicating that reduced Lactobacillus and increased Bifidobacterium might play a key role. This is supported by earlier investigations revealing positive correlation between Lactobacillus and obesity [3,83], and negative association between Bifidobacterium and obesity [84,85]. The remains faecal suspensions from Au@P-NPs-treated...
Fig. 7. Changes in bile acid profile of HFD-fed mice after Au@P-NPs. (a) Profile of bile acids in plasma. Profile of bile acids in faecal samples. The mRNA was isolated from indicated fat tissues of mice and the expression pattern of indicated genes was measured by RT-PCR. (c) Ileum. (d) Liver. (e) eWAT. (f) iWAT. (g) BAT. (h) Significant correlation between the mRNA expression profiles of BAT and iWAT (dark values indicate significant levels). (i) Free glycerol content in plasma. One-way ANOVA repeated measurements with BPHT. *P < 0.05, **P < 0.01 and ***P < 0.001 for CD vs HFD; $P < 0.05, $$P < 0.01 and $$$P < 0.001 for Au@P-NPs vs HFD.
Fig. 8. Re-establishment of germ free (GF) mice with the faecal microbiota of Au@P-NPs-treated animals summarizes the effects of Au@P-NPs on body weight increase and energy expenditure. GF animals were inoculated either with the faecal slurry of HFD-fed animals (HFD recipients) or Au@P-NPs-treated animals (Au@P-NPs recipients) and reserved in metabolic cages with a less-fat containing diet during the early days of PC. Animals were shortly shifted into GF cages and kept under aseptic conditions. (a) body weight increase; (b) energy intake; (c) energy expenditure (the connection among metabolic rate and body weight was stabilized by using body mass^{0.75}). (d and e) Taxonomic gut microbial profile of HFD and Au@P-NPs recipients at phylum and the LTL obtained at indicated days PC. (f) LEfSe calculation of taxa at the LTL likely better discriminates the GM of HFD and Au@P-NPs recipients at days PC. (g) F/B ratio in HFD and Au@P-NPs recipients. (g–n) The abundance of specific bacteria was measured by qPCR analysis and the number of copies of each taxon was stabilized by $3 \times 10^9$ copies of total bacteria. Two-way frequent measurements ANOVA with BPHT applied to assess the significant differences between time points. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. 

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and HFD-fed donor animals were combined and total phenolic content (TPC) in these suspensions was quantified [86]. We noticed 0.104 μg gallic acid equivalent (GAE)/mg of faeces in the slurry prepared from Au@P-NPs-treated stools (Fig. S8j). This content is 1099x lower than the TPC noticed in the aqueous extract of *C. verum* bark (AECV; 114.3 μg GAE/mg) (Fig. S8j), suggesting that the gut microbial community of Au@P-NPs recipients were responsible for the anti-obesity effect and not because of the polyphenols present in the faecal resuspensions.

Polyphenols are well known for their potent antioxidant property [87]. The possible increase in the antioxidant effect, as assessed by DPPH (Fig. S9a) and ABTS (Fig. S9b) radical scavenging assays in mice lumen, was related to the phenolic compounds of *C. verum*. Au@P-NPs might decrease the abundance of facultative anaerobes such as Streptococci and Lactobacilli due to hold back oxygen availability. This assumption is supported by a significant decline in the population of *Lactobacillus* species examined in mice administered with the antioxidant tempol and *Myrciaria dubia* [3,75]. Au@P-NPs acted as superior anti-obesity agents as compared to Au-NPs.

4. Conclusion

This study is the first to provide unequivocal evidence that oral administration of *C. verum* derived bioactives-functionalized AuNPs (Au@P-NPs) increases energy expenditure and alters bile acid profile through gut microbiota in HFD-induced obese mice. Au@P-NPs increased mRNA expression of *UCP1* in the brown adipose tissue, altered plasma bile acid profile, and increased the abundance of *A. muciniphila* and *Bifidobacterium* and decreased the abundance of *Lactobacillus* (Fig. 9). FMT revealed lesser weight increase and higher energy expenditure in the GF mice with re-established the gut microbiota through Au@P-NPs treatment compared to GF animals reconstituted with the faecal suspension of HFD-fed mice. Au@P-NPs showed no toxicity sign in liver, lung, kidney, heart, and pancreas, and no significant alteration in blood serum parameters after 8 weeks of administration. Intestinal microbiota reshaping with Au@P-NPs revealed immense potential as alternatives to existing anti-obesity drugs.

Credit author statement

Vivek K. Sharma: Performed experiments, Data analysis & Writing, Prateeksha: Performed experiments, Data analysis & Writing. Sateesh C. Gupta: Performed experiments, Data analysis & Writing. Brahma N. Singh. Conceptualization, Funding, Writing – Review & Editing. Chandana V. Rao: Performed animal experiments, Data analysis & Writing, Ethical Approval. Saroj K. Barik: Conceptualization, Funding, Writing Review & Editing

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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