Antiobesity and antioxidant effects of a new polyherbal formulation (PHF) in obesity induced Wistar rats

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Obesity is a serious problem and a solution is needed for its treatment and management. Natural products which are more targeted offer an alternative therapy. Objective of the study is to determine the protective effects of a new Poly Herbal Formulation (PHF) against High Fat Diet (HFD) induced systemic oxidative stress in female wistar rats. Detailed chemical investigation was carried out for the polyphenolic rich PHF by preparative HPTLC fingerprinting. Obesitic parameters including anthropometric, physiological and biochemical parameters were evaluated in vivo using female wistar rats. The results of HPTLC preparative fingerprinting analysis chromatogram confirmed the presence of polyphenols such as curcumin and plumbagin in the PHF. In vivo results revealed significant reduction in body weight, BMI, abdominal circumference, lipid profile and levels of enzymic antioxidant values in treatment groups (p < 0.05). The present study suggests that the new PHF has antiobesity effects and showed protective action against oxidative stress and hepatic injury induced as a result of obesity.

Keywords: Obesity, Antioxidants, Inflammation, BMI, Curcuma longa, Macrotyloma uniflorum, Phyllanthus emblica, Plumbago zeylanica.

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Obesity is characterised by abnormal lipid metabolism¹ and excess adipose (lipid storing adipose cell) tissue mass². Several comorbidities such as hypertension, insulin resistance, hyperinsulinenia, dyslipidemia, glucose intolerance, and microalbuminemia are associated with it. One of the major complications associated with obesity is impaired antioxidant defence system³. Chronic oxidative stress in obesity leads to the development of end organ damage, especially in cardiovascular system and liver as a result of atherosclerosis and non-alcoholic hepatic steatosis⁴. This study is focussed on the indispensable beneficial effects in terms of antioxidant improving capacity in rats exposed to HFD by the newly developed PHF which is a combination of four herbal drugs as follows: Phyllanthus emblica L. (fruits), Curcuma longa L. (rhizome), Macrotyloma uniflorum L. (seeds) and Plumbago zeylanica L.

Materials and methods

Herbal drugs Phyllanthus emblica L. (fruits), Curcuma longa L. (rhizome), Macrotyloma uniflorum L. (seeds), and Plumbago zeylanica L. (roots) were identified and authenticated by Dr S Rajan, Field Botanist, Survey of Medicinal Plants and Collection Unit, Central Council for Research in Homeopathy Department of AYUSH, Emerald, Ooty. All chemicals used for the study were obtained from Sigma –Aldrich Chemical Co. Ltd and S.D. Fine Chem Limited, India.

Preparation of the polyherbal formulation (PHF) extract

The herbal drugs were dried and powdered. A ratio of 6:3:12:1 (by weight) of each of the herbs were taken (based on Ayurvedic Pharmacopoeial limits mentioned) and mixed to get a homogenous PHF. They were stored in air tight bottles for subsequent studies. The powdered PHF was charged into Soxhlet apparatus and successive extraction was done using the following solvents: petroleum ether, chloroform, ethanol, and water (1:10 w/v). The dried residue was collected and stored in opaque glass bottles for further studies. The percentage yield was calculated for each of the solvent extracts. Detailed chemical investigation of the ethanolic extract (percentage yield obtained for ethanolic extract was higher than the other solvent extracts used) with respect to polyphenolic content was done using preparative HPTLC according to standard protocols⁵,⁶.

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Preparative HPTLC fingerprinting analysis

Detection of curcumin and plumbagin

TLC plates of size 20 x 10 cm were used. 300 µl of test solution were loaded as 50 mm x 3 spots. The mobile phase used was CHCl₃: Methanol: water (4.8:0.2: 0.2). The spots were developed inside a twin chamber that was saturated with solvent phase. The individual spots were scrapped, suspended in methanol and stored individually in Eppendorf tubes.

Experimental protocol

Animals and diets

Thirty female Wistar rats (100-150 gm) were purchased from Sri Venkateshwara Enterprises, Bangalore. They were maintained under standard conditions (temperature: 22±2 °C, relative humidity of 30-70 % and 12 hrs light /dark cycle). They had free access to water ad libitum and were adapted to standard pellet diet for a week. The experimental protocol was approved and ethical clearance was obtained from the Institutional Animal Ethics Committee (IAEC)-KMCRET/PhD/10/2011. Commercial pellet chow diet (VRK Solutions, Bangalore) was fed to the untreated control group. Obesity was induced by High Fat Diet (HFD) with slight modifications of Srinivasan et al., (2005). Acute oral toxicity of PHF was carried out as per OECD test guidelines 423 for testing of chemicals in female wistar rats.

Experimental groups

A total of 30 rats (female Wistar rats) were randomly assigned into two groups: Group I animals received normal pellet chow diet and Group II animals received high fat diet (HFD) with slight modifications of Srinivasan et al., (2005). Acute oral toxicity of PHF was carried out as per OECD test guidelines 423 for testing of chemicals in female wistar rats.

Group I: Untreated control (commercial pellet chow diet).
Group II: High Fat Diet (HFD) + normal saline.
Group III: HFD+ Std. (L-Carnitine) (250 mg/kg/day body weight, per oral route).
Group IV: HFD + PHF (200 mg/kg body weight, per oral route).
Group V: HFD+ PHF (400 mg/kg body weight, per oral route).

Anthropometric parameters

Parameters for indicators of obesity such as body weight gain, Body Mass Index (BMI), and Abdominal Circumference (AC) were measured. All the measurements were done after anaesthetization of rats by inhalation of mild dose of diethyl ether.

Physiological measurements

The food intake and the mean of food consumption per rat was calculated (gm/day/rat). Determinations for food consumption were made at the beginning of the experiment, once in a week, after the fattening period, and at the end of the experimental period. The calorie intake was calculated based on the amount of food consumed and the corresponding constants.

Sample preparation and biochemical studies

At the end of the experimental period, blood samples were collected from the over-night fasted animals under ether anaesthesia. A clear, non-haemolysed supernatant serum was collected and stored at -20 °C. Total Cholesterol (TC), High Density Cholesterol (HDL-C), Low Density Lipoprotein Cholesterol (LDL-C), Very Low Density Lipoprotein Cholesterol (VLDL-C), and Triglycerides (TG) was determined using commercial kits (Span Diagnostics Pvt. Ltd. India).

The following formulae were applied to calculate serum LDL-C value, Atherogenic Index (AI) and coronary risk index (CRI) respectively:

\[ \text{LDL-C} = \text{TC} - (\text{HDL-C} + \text{TG}/5) \]
\[ \log (\text{TG}/\text{HDL-C}) \]
\[ \text{ratio of TC and HDL} \]

Low-density lipoprotein (LDL) cholesterol concentrations are most commonly estimated by the formula:

\[ \text{LDL cholesterol} = \text{total cholesterol} - [\text{triglycerides (TG)/5 + high-density lipoprotein cholesterol}], \text{although alternative factors such as TG/6 have also been used.} \]

Using standardized, automated, enzymatic lipid assays, plasma samples from normal and dyslipidemic adults, to compare LDL cholesterol concentrations obtained after ultracentrifugation with those calculated by several such methods (i.e., TG/4-TG/8). or TG concentrations less than or equal to 0.50 gm/L, TG/4 agreed best with the direct assay; for TG of 0.51-2.00 gm/L, TG/4.5 was best; and for TG of 2.01-4.00 gm/L, TG/5 was best. At TG greater TG were than 4.00 gm/L, none of the factors tested allowed a reliable estimate of LDL cholesterol.

Animals were then euthanized and the liver samples were separated immediately, washed in ice cold saline followed by pH 7.4 buffer and blotted with filter paper. The weight was recorded in gram and the tissue homogenate (10 %) was prepared with 0.025 M.
Tris-HCl buffer, (pH 7.5). The levels of Reactive Oxygen Species (ROS) are controlled by antioxidant enzymes like SOD (Super Oxide Dismutase), CAT (Catalase), GPx (Glutathione peroxidase), GR (Glutathione Reductase), and GST (Glutathione-S-Transferase) were estimated by standard protocols.

### Statistical analysis
The results are expressed as mean ± SD. Mean difference between the groups were analysed by Analysis of Variance (ANOVA) followed by Bonferroni’s post test using Graph Pad Prism 5.01 software. p values, *p < 0.05, **p < 0.01, ***p < 0.001 were considered significant.

### Results
The ethanolic extracts of the PHF were chosen for the study after the standardisation protocol mainly based on the highest percentage yield and total phenolic content confirmed. The phenolic constituents were screened, separated and quantified by HPTLC fingerprinting analysis. Yellowish blue coloured fluorescent zone and black coloured quenching zone at UV 366 nm mode were present in the tracks, it was observed from the chromatogram after derivatization, which confirmed the presence of curcumin and plumbagin in the given standards and in the PHF. A common solvent phase (CHCl₃: H₂O: CH₃OH- 9.6: 0.4: 0.4) was optimized for curcumin and plumbagin and showed two spots, i.e., Spot 1- Rₐ 0.96 (167 mg) and Spot 2-Rₐ 0.80 (76 mg) (Fig. 1). Acute toxicity study indicated no mortality or any clinical sign of toxicity in animals treated with PHF even at a maximum dose of 2000 mg/kg. Thus, in accordance with the GHS Classification and Labelling of chemicals, PHF was classified as Category 5.

Significant changes in physiological and anthropometric parameters were observed (Tables 1&2; Figs. 2(i-ii); 3 (i-ii); 4 (i-v). A significant decrease in

Table 1 - Effect of PHF and L-Carnitine on BMI, AC and on Organ/tissue weights in female wistar rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>BMI (gm/cm²)</th>
<th>AC (cm)</th>
<th>Liver wt.(gm)</th>
<th>Mesenteric fat (gm)</th>
<th>Uterine fat (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.71±0.07</td>
<td>17.79±0.19</td>
<td>6.04±0.49</td>
<td>1.01±0.09</td>
<td>0.52±0.018</td>
</tr>
<tr>
<td>II</td>
<td>1.14±0.05</td>
<td>21.55±0.84</td>
<td>8.68±0.40</td>
<td>6.55±0.44</td>
<td>1.58±0.03</td>
</tr>
<tr>
<td>III</td>
<td>1.01±0.05***</td>
<td>18.55±1.8***</td>
<td>6.16±0.19***</td>
<td>2.96±0.10***</td>
<td>1.24±0.02**</td>
</tr>
<tr>
<td>IV</td>
<td>1.01±0.05***</td>
<td>18.96±0.31***</td>
<td>7.00±0.34***</td>
<td>2.79±0.77***</td>
<td>1.34±0.02**</td>
</tr>
<tr>
<td>V</td>
<td>1.01±0.06***</td>
<td>18.78±0.42***</td>
<td>7.50±0.28***</td>
<td>3.05±0.32***</td>
<td>1.33±0.03**</td>
</tr>
</tbody>
</table>

Footnotes:
All values are expressed as mean ± S. D, (n=6 rats/group). *p< 0.05, **p<0.01, ***p<0.001 as compared with the Group II (ANOVA, followed by Bonferroni’s multiple comparison test).

Table 2 - Lipid profile, AI and Coronary risk index of female wistar rats after treatment with PHF and L-Carnitine

<table>
<thead>
<tr>
<th>Biochemical Indices</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>TG</td>
<td>83.42±3.66</td>
</tr>
<tr>
<td>TC</td>
<td>76.39±3.30</td>
</tr>
<tr>
<td>HDL</td>
<td>47.75±1.17</td>
</tr>
<tr>
<td>LDL</td>
<td>11.96±3.38</td>
</tr>
<tr>
<td>VLDL</td>
<td>16.68±0.73</td>
</tr>
<tr>
<td>AI</td>
<td>0.24±0.02</td>
</tr>
<tr>
<td>CRI</td>
<td>1.59±0.07</td>
</tr>
</tbody>
</table>

Footnotes:
All values are expressed as mean ± S. D, (n=6 rats/group). *p< 0.05, **p<0.01, ***p<0.001 as compared with the Group II (ANOVA, followed by Bonferroni’s multiple comparison test).
liver anti-oxidant enzymes was observed in the HFD treated group in comparison to the untreated control group. However, significant increase in the levels of the enzymes was observed in the treatment groups (III, IV, V) (p < 0.05). This indicates that treatment with the PHF showed protection against oxidative stress induced by the depletion of enzymic antioxidants.

Discussion

The high percentage yield of the ethanolic extract of PHF indicated a high amount of polyphenols (phenolic acids and flavonoids). Polyphenols are a major class of therapeutic agents known for the antioxidant activity with less adverse effects. Laboratory studies indicate that the antiobesity effects of polyphenol rich diets may be attributed to the ability of polyphenols to interact, directly or indirectly, with adipose tissues. The current results showed that body weight increased significantly in the HFD group compared with the normal group (Fig. 3 (i-ii)) which is associated with increased food intake and thus higher calorie intake. The total calorie intake was higher in group II which implied that was contributed by the HFD. One gm of standard rat chow contributed to 3.8 kcal. Meanwhile, 1 gm of HFD yields 5.5 kcal. With increase in calorie intake, there is more deposition of lipid, which contributes to the development of obesity and metabolic syndrome. Three indicators of obesity such as percentage of body weight gain, BMI, and AC were measured and found to be higher in group II. The normal BMI for female wistar rats was in the range of 0.66 ± 0.01 gm/cm² to 0.74 ± 0.05 gm/cm². Our study showed that, following six weeks of consumption of HFD group had higher BMI (0.97 gm/cm²) as compared to the control group. One of the major reasons for HFD induced obesity is due to a positive energy balance leading to an increase in visceral fat deposition or abdominal obesity. This abdominal obesity particularly, a large increase in periareal visceral adipose tissue mass, suggests that the excess energy leads to the buildup of adiposity. A cholesterol rich
and energy dense diet is a major factor for the development of atherosclerosis. From our present study, it can be concluded that the lipid profile levels significantly increased atherogenic index with the induction of a high density diet. Atherogenic index which is an indicator of atherosclerosis development was significantly reduced in treated groups.

Significant increase in the enzymic antioxidant (p < 0.05) (CAT, SOD, GST, GR, GPx) concentrations in the animal groups treated with the two doses of PHF indicated that the Reactive Oxygen Species (ROS) were generated as a result of HFD and obesity. This suggests that the above enzymes play a vital role in protecting the organism against the damaging effects of the radicals and also critical for maintaining optimal cellular and systemic health and well being. The liver plays a central role in the lipoprotein metabolism including enzymes and receptors involved in lipoprotein metabolism such as HMG Co A reductase and LDL receptors. Antioxidants are known to effectively prevent this kind of cellular damage. The lipoprotein structure is maintained for the cellular uptake of serum lipids from the blood and oxidative state destructs this ability. We obtained significant depletion of enzymatic antioxidant levels in the liver after treatment with L-Carnitine and PHF comparing to HFD induced obese and normal rats. Overall, the PHF was more efficient in increasing antioxidant levels which could explain improvement in histological and biochemical parameters of liver necrosis due to PHF treatment.

**Conclusion**

The US Food and Drug Administration (FDA) recommend developing plant derived drugs which are excellent alternative to synthetic drugs as they can be developed at much faster rate and cheaper prices. Because obesity is a serious problem, an effective and strategic solution is needed for its treatment and management. It is the need of the hour to develop products from natural sources that are more targeted, cost effective and provide a realistic alternative therapy to control obesity. Obesity is associated with complications such as insulin resistance, dyslipidemia, cardiovascular disease (CVD), etc. Both L-Carnitine and PHF have shown significant decrease in liver enzymes, and increase in antioxidant enzymes. Obesity and its associated problems in this study could be ameliorated in different degrees by using L-Carnitine or PHF extract suggesting that PHF has good antioxidant property and restoring normal metabolism in the liver. This work demonstrated that our PHF developed has the potential to mitigate the level of obesity indices and its associated oxidative stress. In the present scenario, chemical compounds and products derived from innumerable number of medicinal plants have become of great interest owing to their wide range of applications. Medicinal plants provide the richest source of drugs which form the basis for the different traditional systems of medicine, folk medicine, modern medicines, nutraceuticals, functional foods, pharmaceutical intermediates and chemical entities for synthetic drugs. Herbal medicines are prepared from parts of herbs or whole herbs either as single or in combinations or as purified compounds such as phytochemicals. Synergistic effects using multiple products with similar targets or products that act on multiple targets are proved to achieve desirable therapeutic effects.

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Conflict of interest
The authors report that they have no conflicts of interest.

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