Original article

Evaluation of antioxidant, anti-inflammatory and adipocyte differentiation inhibitory potential of Ziziphus mauritiana bark extract


APT Research Foundation, Pune 411 041, India

ABSTRACT

Introduction: Ziziphus mauritiana is a shrubs belonging to family Rhamnaceae and distributed in warm temperature zone from western Africa to India. Earlier, anti-cancer, anti-inflammatory and anti-diabetic activities were attributed to Z. mauritiana.

Materials and methods: The antioxidant activity of chloroform, ethanol and aqueous extracts of Z. mauritiana bark was assessed by ABTS + decolourization assay. The anti-inflammatory activity was studied in Wistar rats by Carrageenan induced paw edema assay. The adipocyte differentiation (in 3T3-L1 adipocyte cell) and glucose uptake (in CHO-HIRC-mycGLUT4e GFP cells) assay of Z. mauritiana bark aqueous extract was performed.

Results: The chloroform, ethanol and aqueous extracts showed good antioxidant activity. The anti-inflammatory activity (27.83% reduction in inflammation) was observed with chloroform extract at the concentration of 200 mg/kg body weight when administered orally in rats. The aqueous extract showed reduction in number and size of the oil droplets in 3T3-L1 adipocyte cell cytoplasm which was found to be dose dependent. The aqueous extract stimulated the glucose uptake in the CHO-HIRC-mycGLUT4e GFP cells which was comparable to insulin.

Conclusion: The chloroform, ethanol and aqueous extracts showed good antioxidant activity. The anti-inflammatory potential was shown by chloroform extract only. The adipocyte differentiation inhibition and glucose uptake in CHO-HIRC-mycGLUT4e GFP cells was induced by aqueous extract.

Copyright © 2013, Phcog.Net, Published by Reed Elsevier India Pvt. Ltd. All rights reserved.

1. Introduction

On a global scale, obesity has reached epidemic proportions and is a major contribution to the global burden of chronic disease such as type 2 diabetes, cardiovascular complications and disability.1

Two different types of the drugs are currently available in the market for the treatment of obesity. One of these is Orlistat (xenical) and other is sibutramine (reductil). Orlistat reduces intestinal fat absorption through inhibition of pancreatic lipase, whereas Sibutramine is an appetite suppressant.2-3 Both these drugs have side effects, including increased blood pressure, dry mouth, constipation, headache and insomnia. A number of anti-obesity drugs are currently undergoing clinical development, including centrally acting drugs, drugs targeting peripheral episodic satiety signals, drugs blocking fat absorption and human growth hormonal fragments.1

A variety of natural products also have been proposed as pharmacological treatments for obesity preferably as many synthetic drugs are potentially toxic and are not free of side effects. Several medicinal plants have been reported to induce body weight reduction and prevent diet induced obesity.4 Amongst these plants, Z. mauritiana and Ziziphus jujuba are shrubs belong to family Rhamnaceae distributed in warm temperature zone from western Africa to India. Earlier, anti-cancer, anti-inflammatory and anti-diabetic activities were attributed to Z. mauritiana.5-7 Recently anti-obesity activity of Z. jujuba leaf extract has been demonstrated in rats fed on high fat diet.8 In previous studies Z. mauritiana bark powder did show weight reduction and loss of peritoneal fat pads in high fat diet induced obese rats.9 In the present studies we have standardized bioassays to determine antioxidant, anti-inflammatory and lipase inhibition activity of different extracts of bark of Ziziphus mauritiana. The aqueous extract was evaluated for its adipocyte differential activity and insulin mimetic activity in vitro assays.

2. Material and methods

2.1. Chemicals

The ABTS, IBMX, Insulin, Dexamethasone, Cerulein, Glucose and Carrageenan were obtained from Sigma—Aldrich Chemical Co,
St. Louis, USA. All the other chemicals used were of Analytical grade and purchased from local supplier. Tritiated 2-DG (15 Ci mmol\(^{-1}\)) was obtained from Amersham Biosciences (Piscataway, NJ, U.S.A.). DMEM and FBS, were purchased from Life Technologies Inc. (Rockville, MD, U.S.A.). Cultures of 3T3-L1 preadipocytes (ATCC no. CL-173) were obtained from National Centre for Cell Science, Pune, India.

2.2. Animals

Male Wistar rats (100–150 g) were used to determine anti-inflammatory activity of chloroform, ethanol and aqueous extract of bark of *Ziziphus mauritiana*. The animals were maintained under standard laboratory conditions at an ambient temperature of 22 ± 2 °C having 50 ± 5% relative humidity with 12 h light and dark cycle. Animals were fed a standard laboratory diet with water *ad libitum*.

The Institutional Animal Ethical Committee approved the experimental protocols as per CPCSEA guidelines through the research project no. 81.

2.3. Plant materials

*Z. mauritiana* (ZM) bark was collected in the month of November 2010 from a full grown tree in the Solapur District of Maharashtra state, India and was authenticated by Botanical Survey of India, Pune under the certificate no. BSI/WRC/Tech./2011. The bark was cleaned, dried and ground to a coarse power with the help of pulverizer.

2.4. Preparation of extracts

The coarse ZM bark powder (ZMBP) was kept in Soxhlet apparatus. Chloroform, ethanol and aqueous extracts were obtained by serial extraction with respective solvents. The extracts obtained were concentrated in rotary evaporator under vacuum and their percent yields were determined.

2.5. Phytochemical study

Qualitative analysis of ZMBP extracts for the presence of flavonoids, saponins, tannin, steroids was carried out as per the method described by Khandelwal, 2008.\(^{10}\)

2.6. Determination of antioxidant potential by in vitro ABTS + decolorization assay

Antioxidant effect of extracts was studied using ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) radical cation decolorization assay according to the method of Re et al, 1999.\(^{11}\) ABTS radical cations (ABTS+) were produced by reacting ABTS solution (7 mM) with 2.45 mM ammonium persulphate. The mixture was allowed to stand in dark at room temperature for 12–16 h before use. The absorbance was read at 735 nm in a spectrophotometer. The percent inhibitory activity of the plant extract was determined by the following formula and the results were compared with ascorbic acid as standard.

\[
\text{% inhibition} = \left(\frac{\text{Abs control} - \text{Abs test}}{\text{Abs Control}}\right) \times 100
\]

The concentration equivalent to ascorbic acid was calculated by plotting the values of the test extracts on the standard curve of ascorbic acid.

### 2.7. Determination of anti-inflammatory potential of ZMBP extracts

The assay based on ability of anti-inflammatory agent to inhibit the edema produced in the hind paw of the rat after injection of carrageenan (phlogistic agent).\(^{12}\)

Male Wistar rats weighing around 100 g–150 g were used for this assay. Five groups of six animals each were starved overnight. Group 1 Control, group 2 Standard drug (Indomethacin 10 mg/kg body weight), group 3, group 4 and group 5 animals administered chloroform, ethanol and aqueous extracts orally at a dose of 200 mg/kg body weight respectively. One hour after dosing, the rats were challenged by a subcutaneous injection of 0.2 ml of 1% solution of carrageenan into the sub planter region to induce edema. The paw edema was measured with a vernier caliper before carrageenan injection and then at 0, 1, 2, 4 and 6 h after carrageenan injection. The difference between the initial and subsequent values gave the actual edema which was compared with the control animals. The present inhibition of inflammation was calculated using the following formula.

\[
\text{% inhibition} = \left(\frac{C - T}{C}\right) \times 100
\]

where C represents mean edema in Control and T represents mean edema in group treated with standard drug or test drug.

### 2.8. Adipocyte differentiation assay

In brief, 3T3-L1 preadipocyte were seeded as 5 × 10^3 cells/well in 24 well plate. After reaching the confluence (48 h) the preadipocytes were induced by IBMX + insulin (10 mg/ml) medium to differentiate to adipocytes for another 48 h. The aqueous extract of ZM (ZBEaq) and Cerulolin (a known anti-differentiating agent) were added at different concentration and incubated at 37 °C under humidified 5% CO₂ atmosphere. The medium was then replaced every 2 days with DMEM containing 10% FBS and 5 μg/ml insulin. On day 6, the medium was completely removed for triglyceride estimations. Formaldehyde was added slowly to each well and kept for 30 min at room temperature. Formaldehyde was then aspirated and Oil Red O solution (0.5 g/100 ml Isopropanol) was added in each well and incubated for 1 h at room temperature. The stain was removed completely and washed with distilled water twice. After drying the plate, photographs were taken. The Oil Red O stained oil droplets were extracted in Isopropyl alcohol and absorbance was determined spectrophotometrically at 520 nm.\(^{13}\)

### 2.9. GLUT4 translocation assay in CHO-HIRC-mycGLUT4e GFP cells

CHO-HIRC-mycGLUT4e GFP cells (1 × 10^4 cells/well) were plated in 35 mm plates and serum starved by incubating them in F-12 medium containing 1 mg/ml BSA for 3 h, before addition of insulin or ZBEaq for 10 and 30 min, respectively. GLUT4 translocation was visualized by tracking the movement of GLUT4 associated GFP fluorescence from perinuclear space to the plasma membrane by employing cooled charge coupled device (CCD) camera attached to fluorescent microscope.\(^{14}\)

### 2.10. Effect of ZBEaq on glucose transport in CHO-HIRC-mycGLUT4e GFP cells

3H-2-deoxy-o-glucose (2-DG) uptake was measured as described by Vijaykumar 2005.\(^{15}\) In brief, CHO-HIRC-mycGLUT4e GFP cells (2 × 10^3 cells/well) grown in 12 well plate were treated with insulin or vehicle control or ZBEaq for 10 and 30 min, respectively. The glucose uptake was initiated by adding 0.1 mM 2-deoxy glucose.
containing 0.5 µl/ml of labeled 2-DG for 10 min at 37 °C. The reaction was terminated by keeping the cells on ice and washing 3 times with ice cold PBS containing 20 mM 2D glucose. The cells were solubilized with 0.1% SDS sodium dodecyl sulfate. The lysate was transferred on blotting paper and allowed to dry at 37 °C overnight. Scintillation fluid (1CN) 1.0 ml was added and radioactivity incorporated into cells was quantified in a Liquid Scintillation counter (Packard, Albertville, MN USA).

2.11. Statistical analysis

The data expressed as mean ± SD. Statistical comparisons made using student t-test and p-value <0.05 were considered significant.

3. Results

3.1. Phytochemical analysis

The Phytochemical analysis of various extracts of Z. mauritiana bark is summarized in Table 1. The ethanol and aqueous extracts showed apparently similar compositions of tannins, saponins and flavonoids.

3.2. Antioxidant activity of ZMBP extracts

The rate of decolorisation of the bluish green ABTS+ radicals revealed the antioxidant content in the plant extract which was calculated and compared with standard ascorbic acid. The chloroform, ethanol and aqueous extract of bark of Z. mauritiana all showed appreciable inhibition of ABTS+ radical cation as shown in Fig. 1.

3.3. Anti-inflammatory activity of ZMBP extracts

At 6 h, chloroform extract showed better anti-inflammatory activity, i.e. 27.83% inhibition as compared to the control group. Inhibition values obtained were comparable with Indomethacin a known anti-inflammatory agent i.e. 34.32% inhibition. Anti-inflammatory activity of ethanol and aqueous extracts of ZMBP did not show appreciable reduction in paw edema of treated rats (Table 2).

3.4. Adipocyte differentiation in the presence of ZBEaq

Preadipocyte (3T3-L1) cells differentiate into adipocytes upon addition of IBMX + insulin induction medium. When ZBEaq was added to the adipocyte culture in the presence of induction medium, preadipocytes remained largely undifferentiated as shown by Oil Red O staining of the treated cells which was comparable to the Cerulinin (Fig. 2a and b). Number and size of the oil droplets in cell cytoplasm were reduced in presence of ZBEaq, which was found to be dose dependent.

3.5. GLUT4 translocation and ZBE induced glucose uptake

In subsequent studies, ZBEaq found to induce GLUT4 translocation as comparable to insulin in CHO-HIRC-mycGLUT4e GFP cells. ZBEaq stimulation of cells expressing GLUT4 transfer from internal compartment to peripheral membrane with resultant increase in the rate of glucose transport was observed as evident in Fig. 3.

4. Discussion

A variety of natural products including crude extracts and isolated compounds from plants, can induce body weight reduction and prevent diet induced obesity.14 These natural products exhibited many potential anti-obesity mechanisms such as a) lipase inhibitory effect15 b) suppressive effect on food uptake16 c) stimulatory effect on energy expenditure17 d) inhibitory effect on adipocyte differentiation18 e) regulatory effect on lipid metabolism.19 Earlier we have reported anti-obesity activity of Z. mauritiana bark in high fat diet induced obese rats by reducing body weight gain, increasing fecal fat mass and decreasing insulin resistance.9 In the present studies we have carried out extraction of Z. mauritiana bark powder using different solvents i.e. chloroform, ethanol and water. Phytochemical analysis showed that chloroform extract contains mainly steroids whereas ethanol and aqueous extract contains tannins as catechin, saponins and flavonoid as quercetins in different amounts. All three extracts showed appreciable antioxidant activities in ABTS+ radical cation assay. Chloroform extract of Z. mauritiana bark also showed appreciable anti-inflammatory activity. The ethanol and aqueous extracts did not show an anti-inflammatory activity.

Dietary fat is not directly absorbed by the intestine unless fat has been subjected to the action of pancreatic lipase.1 Therefore pancreatic lipase is one of the most widely studied mechanisms for determining anti-obesity potential of any natural product. In our

![Fig. 1. Antioxidant activity of ZMBP extracts.](image-url)
previous studies Z. mauritiana fed obese rats showed significant decrease in pancreatic lipase activity at different dosages.9

Adipogenesis is another common feature of obesity in several studies, screening for anti-obesity products have focused on the processes of adipocyte proliferation and differentiation. 3T3-L1 preadipocyte cells are currently used as in vitro model for the study of obesity.21 While differentiating into adipocytes from preadipocytes in presence of IBMX and insulin, the cells accumulate lipids intracellularly in the form of lipid droplets. These oil droplets can be measured by staining with Oil Red O solution. In our studies when ZBEaq was added to the preadipocyte in the presence of IBMX and insulin, preadipocytes remained largely undifferentiated as shown by Oil Red O staining of the treated cells. Oil droplet formation and their sizes were reduced significantly and found to be dose dependent.

Insulin mimetic activity of aqueous extract of Z. mauritiana was studied using glucose transporter 4 (GLUT4) translocation to cell membrane leading to glucose uptake using CHO-HIRC-muc-GLUT4eGFP cells. GLUT4 translocation was visualized by tracking the movement of GLUT4 associated GFP fluorescence from perinuclear space to the plasma membrane by employing cooled charge coupled device (CCD) camera attached to fluorescent microscope which ultimately lead to increased glucose uptake.14 These results indicated that ZBEaq inhibited the preadipocytes differentiation induced by IBMX + insulin. Also it was puzzling that ZBEaq showed insulin like glucose transport stimulatory activity in CHO-HIRC-muc-GLUT4eGFP cells. Similar observations were reported by Liu 2005,21 where tannic acid was found to stimulate glucose transport and inhibit adipocyte differentiation in 3T3-L1 cells.

Several natural products have been reported earlier to possess single or multifunctional anti-obesity mechanisms including inhibition of lipid and carbohydrate absorption and acceleration of lipid metabolism and energy expenditure by dietary supplementation of Nelumbo nucifera leaves extract.22 Whereas aqueous extract of Hibiscus sabdariffa containing mainly anthocyanins has exhibited many potential anti-obesity mechanisms, including anti hyperglycemic effects, plasma cholesterol level reduction, gastric and pancreatic lipase inhibition, thermogenesis stimulation, inhibition

<table>
<thead>
<tr>
<th>Group</th>
<th>Paw (edema) thickness in mm</th>
<th>% inhibition in paw edema at 6 h compared to control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before carrageenan injection</td>
<td>After carrageenan injection</td>
</tr>
<tr>
<td>1 Control</td>
<td>Mean 4.08</td>
<td>6.06</td>
</tr>
<tr>
<td></td>
<td>±SD 0.41</td>
<td>0.33</td>
</tr>
<tr>
<td>2 Chloroform</td>
<td>Mean 3.74</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>±SD 0.17</td>
<td>0.75</td>
</tr>
<tr>
<td>3 Ethanol</td>
<td>Mean 3.66</td>
<td>5.81</td>
</tr>
<tr>
<td></td>
<td>±SD 0.05</td>
<td>0.48</td>
</tr>
<tr>
<td>4 Aqueous</td>
<td>Mean 3.55</td>
<td>4.37</td>
</tr>
<tr>
<td></td>
<td>±SD 0.33</td>
<td>0.80</td>
</tr>
<tr>
<td>5 Standard</td>
<td>Mean 3.45</td>
<td>5.81</td>
</tr>
<tr>
<td></td>
<td>±SD 0.05</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Note: All the powdered extracts were suspended in corn oil as a vehicle.

*a* Statistically significant at *p* < 0.05.

**Statistically significant at *p* < 0.001.

Fig. 2: a: Adipocyte differentiation assay % reduction value. b: Photographs of adipocytes at 40× magnification.
Fig. 3. a: Video image analysis of Glut4 translocation assay. b: Glucose uptake by CHO-HIRC-mycGLUT4e GFP cells.
of lipid droplets accumulation in fat cells and fatty acid synthase inhibition. *Garcinia cambogia* extract (active component, hydroxycitric acid) has also displayed multifunctional activity.23

As *Z. mauritiana* aqueous extract is a mixture of tannins saponins and flavonoids, its multifunctional anti-obesity mechanisms such as reduction in weight gain, reduction in insulin resistance, loss of fat through faeces and inhibition of lipid droplet accumulation in fat cells which we have observed earlier together with present studies could be a synergistic effect of mainly tannins and flavonoids.

**Conflicts of interest**

All authors have none to declare.

**Acknowledgment**

We thank Vijayakumar Maleppillil Vavachan and Dr Manoj Kumar Bhat, of National Centre for Cell Science, Pune, India, for assisting to perform experiments using CHO-HRC-myc-GLUT4eGFP cells.

**References**