EVALUATION OF ANTIADIPOGENESIS ACTIVITIES OF SELECTED SPECIES OF CARALLUMA R. BR., BOUCEROSIA WIGHT & ARN. AND PREGNANE STEROID ON CELL LINES.

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ABSTRACT: Background: The study was designed to determine antiadipogenesis properties of crude methanolic extracts from four species of Caralluma R.Br. such as Caralluma adscendens (Roxb.) R. Brown var. attenuata (Wight) Grav. & Mayur. (CAA), Caralluma adscendens (Roxb.) R. Brown var. fimбриata (Wall.) Gravely & Mayur. (CAF), Caralluma stalagmifera C.E.C. Fisch. (CS) and Caralluma stalagmifera C.E.C. Fisch. var. longipetala Karupp. & Pull. (CSL) and as well as two species of Boucerosia Wight & Arn. such as Boucerosia lasiantha Wight. (BL) and Boucerosia umbellata (Haw.) Wight & Arn. (BU) at intervarietal and interspecific levels along with bioactivity studies. Methods: In vitro inhibitory activity of adipogenesis and facilitating activity on adipolysis were evaluated in 3T3 L1 adipocytes. Results: The percentage anti adipogenesis (50 µg/ml ) was also observed with values of 15.29%, 4.20%, 12.27%, 21.85%, 0 and 9.75% respectively. The percentage inhibition of pregnane steroid in adipogenesis of 3T3 L1 preadipocytes by 1 and 5 µg/ml were 21.22% and 35.97% respectively. Discussion: Four of them are effective in inhibiting adipogenesis. When compared to methanolic extracts of Caralluma and Boucerosia species, pregnane steroid was shown to be potential in invitro antiadipogenesis, and showed significant variations in different cell lines at different concentrations.

INTRODUCTION: Obesity is a global health problem and current research on screening antiobesity agents from medicinal plants focuses on molecular regulation of triglyceride synthesis and to reduce fat storage, fat synthesis, size and differentiation of adipocytes as well as proliferation of preadipocytes; to increase lipolysis and fat oxidation. 3T3 L1 cell line is an in vitro convenient model system for research related to obesity ¹.

Keywords: Caralluma, Boucerosia,pregnan steroid, antiadipogenesis and cell lines.

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Flavonoids play an important role in the inhibition of lipogenesis related enzymes in 3T3 L1 cell differentiation for obesity prevention. Quercitin and polyphenols inhibited 3T3 L1 differentiation ², ³ and procyanidin from grapes increased lypolysis ⁴. Caralluma and Boucerosia belong to the family Asclepiadaceae, distributed in Africa, Spain, Saudi Arabia, Middle East, Pakistan and India. The species of Caralluma found in India are eaten raw as well as in cooked form and became a part of traditional system of the country. The medicinal activities of the genus Caralluma include carminative, febrifugal, anthelmintic, antirheumatic, anti-inflammatory, anti-nociceptive and anti-oxidant effects.
Caralluma acts as appetite suppressant and stimulant of central nervous system. The medicinal properties of Caralluma and Boucercosia are due to pregnane group of glycosides contained in them. Pregnanediol glycosides are the condensation products of sugar and non-sugar compounds along with ring structure ⁵. Some of the pregnane glycosides include caratuberside A, caratuberside B and boucercoside I to X ⁶.

Four new pregnane glycosides comprised of genin exhibiting a hydroxymethylene instead of a methyl group at C 19 were isolated with other eight known glycosides and structures were elucidated using 2D-NMR from Caralluma adscendens var. fimbriata ⁷. The extracts of Caralluma fimbriata have shown appetite suppressing, anti obesogenic and antiatherogenic activities in the DIO rat model ⁸. The crude extracts of Caralluma fimbriata standardized to pregnanediol glycosides inhibited proliferation 3T3-L1 preadipocytes by inhibiting the import of cyclin D1 from cytosol into the nucleus, thereby arresting the cell cycle at G1 phase and reported the anti adipogenic mechanism of action of Caralluma fimbriata ⁹.

The aim of the study is to evaluate antidiadipogenesis among selected four species of Caralluma (Asclepiadaceae) such as CAA, CAF, CL and CSF as well as two species of Boucercosia like BL and BU at intravarietal and interspecific levels along with an isolated compound pregnane steroid (an isolated compound from BU and BL). The present investigation has been carried out to differentiate CAF from other five species, as Caralluma adscendens var. fimbriata is commercially used to produce Genaslim capsules for antiobesity.

### Antiadipogenic activity

Mouse 3T3 L1 preadipocyte cell lines were treated with different concentrations of Caralluma fimbriata extract ⁹, Taraxacum officinale ¹⁰ and four natural extracts of Rubi fructus, Cornus fructus, Salix radicis, Geranium nepalense ¹¹ as well as reported that they have potential to inhibit hyperplastic obesity in dose and duration dependent manner, with results comparable to those produced by hydroxyurea. The study is aimed to explore and evaluate antidiadipogenesis in 3T3 L1 adipocytes cell lines of methanolic extracts of Caralluma and Boucercosia

### MATERIALS AND METHODS

The four different species of Caralluma and two species of Boucercosia studied were collected at random, from Gooty, Tadiparthy and Penukonda regions of Ananthapur district, Andhra Pradesh. and were taxonomically identified by comparing with Gamble flora and other taxonomical literature, voucher specimens i.e. VM 46, VM 47, VM 48, VM 49, VM 50 and VM 51 were deposited in Montessori Mahila Kalasala, Vijayawada.

#### Adipogenesis assay with oil red O staining

Cell culture reagents:

- FBS-DMEM, insulin, oil red O, formaldehyde and isopropyl alcohol, DEX-dexamethasone, MIX-3-isobutyl 1-methylxanthine.
- **Equipment:** Microplate reader (Bio – Rad, USA).

#### 3T3-L1 Cell Culture: Preadipocyte maintenance and passage

Cell based adipogenesis inhibition assay was performed ¹², ¹³. The 3T3 L1 Mouse preadipocytes were procured from National centre for cell science, Pune, India. The cells were maintained in Dulbecco’s modified eagle medium containing 10% fetal bovine serum and incubated at 37 °C in 10% CO₂ and grown to 80% confluence.

#### Differentiation of adipocytes

Preadipocytes were grown to confluence in the differentiation medium containing 10% FBS-DMEM, supplemented with 500 nM insulin, 1 μM dexamethasone and 0.5 mM 3-isobutyl 1-methylxanthine for two days. After Dex- MIX treatment, equal number of 3T3 L1 mouse preadipocytes (6 X 10⁴ cells/well) were seeded in each well of 24 well tissue culture plates,
the cells were further maintained in the post differentiation medium containing 10% FBS-DMEM, supplemented with 100 nM insulin in the presence or absence of respective CME test samples and pregnane steroid for further eight days and grown to 90% confluence.

The control cultures received only 0.1% v/v DMSO as the vehicle.

**Preparation of plant extracts:** Stock of *Caralluma* and *Boucerosia* plant extracts (CAA, CAF, CS, CSL, BL and BU) at 100 mg/ml in DMSO were prepared and stored at -0°C until used. Ten-fold serial dilutions (20 µl of plant extracts in 180 µl DMEM) were then prepared from the stock solution to obtain extract working concentrations 25 µg/ml and 50 µg/ml by using DMEM. Pregnan steroid at working concentrations 1 µg/ml and 5 µg/ml was also prepared for this assay. The plant extract solutions were used immediately for cytotoxic assay.

**Oil red O staining:** The intracellular lipid accumulation was measured by staining cells with oil red O. Briefly, the 10% formaldehyde-fixed cells were washed with 60% isopropyl alcohol and the air dried cells were stained with oil red O for 10 min at room temperature. Unbound stain was washed with distilled water. The dye incorporated into the fat vesicles was eluted with 100% isopropyl alcohol, and optical density was read at 550 nm using a microplate reader. Percent inhibition of adipogenesis was expressed in terms of inhibition of fat accumulation or lipid load in the treated cell, calculated by using following formula. Percent inhibition in adipogenesis = (OD in vehicle control – OD in treated well) X 100/ OD in vehicle control.

**RESULTS:** The intracellular lipid accumulation was measured by staining cells with oil red O. Two concentrations (25 µg/ml and 50 µg/ml) of crude extracts of *Caralluma* and *Boucerosia* such as CAA, CAF, CS, CSL, BL and BU were treated against 3T3 L1 preadipocytes. Percent inhibition of adipogenesis was expressed in terms of inhibition of fat accumulation or lipid load in the treated 3T3 L1 preadipocytes. The percentage inhibition of all the selected species in adipogenesis of 3T3 L1 preadipocytes by 25 µg/ml and 50 µg/ml has shown dose dependent responses (Table 1).

<table>
<thead>
<tr>
<th>S. No.</th>
<th><em>Caralluma / Boucerosia</em> species</th>
<th>Concentration of sample (µg/ml)</th>
<th>% inhibition in adipogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Caralluma adscendens</em> var. <em>attenuata</em> (CAA)</td>
<td>25</td>
<td>12.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>15.29</td>
</tr>
<tr>
<td>2</td>
<td><em>Caralluma adscendens</em> var. <em>fimbriata</em> (CAF)</td>
<td>25</td>
<td>-4.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>4.20</td>
</tr>
<tr>
<td>3</td>
<td><em>Caralluma stalagmifera</em> (CS)</td>
<td>25</td>
<td>10.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>12.27</td>
</tr>
<tr>
<td>4</td>
<td><em>Caralluma stalagmifera</em> var. <em>longipetala</em> (CSL)</td>
<td>25</td>
<td>7.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>21.85</td>
</tr>
<tr>
<td>5</td>
<td><em>Boucerosia lasiantha</em> (BL)</td>
<td>25</td>
<td>-25.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>-8.40</td>
</tr>
<tr>
<td>6</td>
<td><em>Boucerosia umbellata</em> (BU)</td>
<td>25</td>
<td>-0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>9.75</td>
</tr>
</tbody>
</table>

The percentage inhibition of pregnane steroid in adipogenesis of 3T3 L1 preadipocytes by 1 and 5 µg/ml were 21.22% and 35.97% respectively (Fig 1). CAA, CSL, CS and BU are effective inhibiting adipogenesis, while CAF and BL were not effective, when 50 µg/ml of crude methanolic extract of *Caralluma* and *Boucerosia* was treated with 3T3 L1 preadipocytes (Table 2).
### TABLE 2: PERCENTAGE INHIBITION IN ADIPOGENESIS OF 3T3 L1 PREADIPOCYTES BY SELECTED SPECIES OF CARALLUMA AND BOUCEROSIA.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Caralluma and Boucerosia species</th>
<th>Percentage inhibition Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Caralluma adscendens var. attenuata (CAA)</td>
<td>15.29±0.94</td>
</tr>
<tr>
<td>2</td>
<td>Caralluma adscendens var. fimbriata (CAF)</td>
<td>4.2±0.7</td>
</tr>
<tr>
<td>3</td>
<td>Caralluma stalagmifera (CS)</td>
<td>12.27±0.89</td>
</tr>
<tr>
<td>4</td>
<td>Caralluma stalagmifera var. longipetala (CSL)</td>
<td>21.85±1.50</td>
</tr>
<tr>
<td>5</td>
<td>Boucerosia lasiantha (BL)</td>
<td>nil</td>
</tr>
<tr>
<td>6</td>
<td>Boucerosia umbellata (BU)</td>
<td>9.75±0.43</td>
</tr>
</tbody>
</table>

All test samples run in triplicates and one way ANOVA test was carried. Values are expressed as mean ± standard deviation (n = 3). The results of ANOVA analysis show significant differences (p<0.05) in the means of inhibition of cellular adipogenesis (%) at 50 µg/ml of *Caralluma* and *Boucerosia* extracts.

**FIG 1: DOSE DEPENDENT PERCENTAGE INHIBITION OF ADIPOGENESIS IN 3T3 L1 PREADIPOCYTES BY PREGNANE STEROID.** *µg/ml represents concentration of pregnane steroid used*

**DISCUSSION:** CAA, CS, CSL and BU were effective in inhibiting adipogenesis, while CAF and BL were not effective when 50µg/ml of crude methanolic extract of *Caralluma* was treated with 3T3 L1 preadipocytes. The metabolic process of adipogenesis and has been well documented with the use of 3T3 L1 cell line\(^{14,15}\).

Under the control of adipose specific regulatory genes, transcriptional cascade gets activated namely peroxysome proliferator-activated receptor-\(\gamma\) (PPAR\(\gamma\)) and CCAAT/enhancer binding protein (C/EBP) during adipocyte differentiation. DEX activates C/EBP and IBMX inhibits soluble cyclic nucleotide phosphodiesterases that lead to increase in cAMP levels intracellularly\(^{16}\), resulting in activation of C/EBP genes and PPAR\(\gamma\), which in turn activate adipocyte specific genes such as fatty acid synthetase, fattyacid binding proteins, leptin and adiponectin.

Insulin and insulin like growth factors activates P13-kinase and Akt activity, leading to adipocyte differentiation. By using the 3T3 L1 model of adipogenesis and adipogenic agents like 0.5 mM IBMX, 1 µM DEX and 10 µg/ml of insulin, four species of *Caralluma* and two species of *Boucerosia* as well as pregnane steroid marker compound were screened.

The methanolic extract of CAA, CAF, CS, CSL and BU inhibited lipid accumulation of 3T3 L1 cells in a concentration dependent manner.

*Caralluma* and *Boucerosia* extracts showed 50% or more adipogenesis inhibition at 25 and 50 µg/ml (**Table 1**). Among six extracts tested, methanolic extract of CAA, CS, CSL and pregnane steroid showed potent inhibitory activity on intracellular lipid accumulation in 3T3 L1 adipocytes.
In the study, it was also observed that the antiadipogenesis effect of pregnane steroid, where the potency was comparatively higher than that of crude extracts. This is the first study to investigate comparative analysis of antiobese characteristics of four species of *Caralluma* and two species of *Boucerosia*, so that the result gave useful information to take another look at differentiation of species. Further investigations are needed to identify the active constituents responsible for antiadipogenesis properties of crude methanolic extracts (CME) from four species of *Caralluma* and two species of *Boucerosia* CAA, CAF, CSL, CS, BL and BU.

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