Research article

Isolation and Characterization of (4Z, 12Z)-Cyclopentadeca-4, 12-Dienone from Indian Medicinal Plant *Grewia hirsuta* and its Hyperglycemic Effect on 3T3 and L6 Cell Lines

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Available online: 1st June 2014

**ABSTRACT**

The current investigation deals with the isolation followed characterization of (4Z, 12Z)-cyclopentadeca-4, 12-dienone from the plant *Grewia hirsuta*, which possesses significant hypoglycemic potential. The plant leaves were extracted with solvent and its chemical components were separated using different chromatographic techniques such as TLC and column chromatography. The separated fractions were tested for their antidiabetic potential by α-amylase inhibitory assay. Further, the structure of the compound with significant α-amylase inhibitory potential was confirmed by spectroscopic methods including UV, FTIR, 1H, 13C NMR and the accurate mass determination was carried out using the Q-TOF mass spectrometer. In addition, the antidiabetic activity of the purified compound was evaluated on 3T3 and L6 cell line by MTT assay. The results of the study prove that the leaves of the selected plant *Grewia hirsuta* contains a potent antidiabetic compound (4Z, 12Z)-cyclopentadeca-4, 12-dienone.

**Keywords:** *Grewia hirsuta*, antidiabetic, hypoglycemic potential, NMR, MTT assay.

**INTRODUCTION**

Diabetes, a metabolic disease, has become a serious problem in today’s society due to its severe and long-term health complications. Cardiovascular disease, retinopathy, neuropathy and nephropathy are the complications associated with Type 2 diabetes. Progress in understanding the metabolic staging of diabetes over the past few years has led to significant advances in the regimen for treatment of this devastating disease. Management of diabetes without any side effects is still a challenge for medical system. This has led the researchers to search for antidiabetic drugs from plants used in the traditional system of medicine. In conventional therapy, Type 1 diabetes is treated with exogenous insulin and Type 2 with oral hypoglycemic agents. The oral hypoglycemic agents currently used in clinical practice have characteristic profiles of serious side effects. Hence there is a need to search for newer antidiabetic agents that retain therapeutic efficacy and are devoid of side effects that could be important sources of such agents. Traditional antidiabetic plants might provide new oral hypoglycemic compounds which can counter the high cost and poor availability of the current medicines/ present day drugs for many rural populations in developing countries. India is well known for its herbal wealth with medicinal plants like *T.graecum*, *A.sativum*, *G.slyvestre* and *S.cumini* and these have been extensively studied for their use in the treatment of diabetes mellitus.

The plant under study is *Grewia hirsuta* (Tiliaceae) which is a traditional herbal medicinal plant. The leaves are used to treat many diseases including cardio toxicity. Hence the current study is aimed at the isolation and characterization of (4Z, 12Z)-cyclopentadeca-4, 12-dienone compound from traditional Indian medicinal plant *Grewia hirsuta*, and its α-amylase inhibitory activity. It also focuses on the MTT assay carried out with 3T3 and L6 cell lines using this purified compound. The invitro antidiabetic efficacy of the crude extract which has already been reported by the authors. The antioxidant and anti proliferative studies of this plant have already been reported.

**Fig. 1:** TLC profile of the purified compound (Rf value 0.88) from *G. hirsuta* developed using the solvent system: Chloroform/methanol (9:1)

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MATERIALS AND METHODS

Solvent extraction: The powdered Grewia hirsuta leaves were sequentially extracted with methanol in a Soxhlet apparatus for 16 h. The solvent was evaporated to dryness under reduced pressure using rotary evaporator (Buchi, Switzerland). The residue was stored in a desiccator until further analysis.

Purification of bioactive metabolites from methanol extract of G. hirsuta

Thin layer chromatography (TLC): TLC was performed on a pre-coated silica gel TLC plates grade F254 (E-Merck, Darmstadt, Germany) to determine the number of compounds present in the given sample. A total of 5 µL of sample was spotted at 1 cm from the bottom of silica gel plates using capillary tubes. Different solvents at various combinations and concentrations were used for metabolites profiling. Development of the chromatogram was done in closed tanks, in which the atmosphere has been saturated with eluent vapor by wetting a filter paper lining. The chromatogram was visualized under UV light 9(365 nm and 254 nm) and iodine vapor. The Rf values of the compounds were calculated using the following formula:

\[
R_f = \frac{\text{distance travelled by the compound}}{\text{distance travelled by the solvent front}}
\]

Silica gel column chromatography: The concentrated crude methanol extract of G. hirsuta was mixed with methanol-silica gel slurry and loaded into a silica gel 100–200 mesh (E-Merck, Darmstadt, Germany) column, packed in low-polar solvent: (the dimension of column was 450 × 30 mm). The column was eluted with stepwise gradient of low/high polar (100:0; 90:10; 80:20; 70:30;
50:50; 30:70; 10:90, v/v) solvents (i.e. chloroform/methanol). Each fraction was checked for the antidiabetic activity by α-amylase inhibition method that has been reported by the author. The active compounds were checked for their purity by TLC. The fraction showing significant antidiabetic activity was further purified using silica gel 230–400 mesh (E-Merck, Darmstadt, Germany) column chromatography. The separation was done by gradient elution with low polar/high polar (gradient from 100% low polar/0% high polar to 0% low polar/100% high polar) using the flow rate of 1 mL/min. One hundred tubes of 10 mL each was collected and then analyzed by TLC. Fractions showing similar spots with same Rf values were pooled and concentrated by a speed-vac under low pressure with an

Table 1: α-amylase inhibitory potential of the obtained fractions

<table>
<thead>
<tr>
<th>S.No</th>
<th>Concentration(µg/ml)</th>
<th>Inhibition (%)</th>
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<tr>
<td></td>
<td>C-1</td>
<td>C-2</td>
</tr>
<tr>
<td>1.</td>
<td>25</td>
<td>33.46</td>
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<tr>
<td>2.</td>
<td>50</td>
<td>40.63</td>
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<tr>
<td>3.</td>
<td>75</td>
<td>48.79</td>
</tr>
<tr>
<td>4.</td>
<td>100</td>
<td>52.73</td>
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Fig. 5: FTIR spectrum of the purified compound from G. hirsuta

Fig. 6: Q-TOF mass spectrum of the purified compound from G. hirsute

Fig. 7: 1H NMR spectrum of the purified compound from G. hirsuta
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Evaporating temperature of 40°C. The active compounds were checked for their purity by TLC. α-Amylase inhibition method: The α-amylase inhibiting activity of the fractions was tested by following the assay method specified by the author7 and the inhibition percentage was calculated using the given formula,

\[
\text{% inhibition} = \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \times 100
\]

Characterization of the Purified Compound

Physical properties: The physical appearance of the purified compound was determined visually. Solubility was checked with methanol, ethyl acetate, chloroform, hexane, DMSO and water. The melting point of the compound was found out using the melting point apparatus (Jayagen Biologics).

Spectral studies: The purified compound was dissolved separately in methanol at 2–10 g/mL concentrations and the UV-Vis spectrum was recorded using a UV-Vis spectrophotometer (Shimadzu, Japan) between 200 and 800 nm. Methanol was used as blank. IR spectrum for the purified compound was obtained by direct sampling method using TENSOR II FT-IR Spectrometer (Bruker, Mumbai, India).

1H NMR and 13C NMR of the purified compound were recorded in CDCl3 with TMS as internal standard solution using 300 MHz Bruker machine (Bruker, MA, USA). The accurate mass determination was carried out using the (Q-TOF) 2 hybrid quadrupole time of flight mass spectrometer available with electrospray ionization (ESI) (Micromass UK Ltd).

Cell Line studies

Cell culture: The 3T3 and L6 cells in Dulbecco’s modified Eagle’s medium (D-MEM) was obtained from NCCS (National Centre for Cell Science, Pune) and incubated for testing their cell viability. 3T3 and L6 cells were grown in D-MEM containing 10% fetal calf serum and 2 mM glutamine. The cells were fed every 2 days during both exponential growth and the differentiation procedure. The cells were maintained in an atmosphere of 10% CO2, 90% air at 37°C.

Adipocyte Differentiation: 3T3 and L6 cells were grown to confluence with D-MEM supplemented with 10% fetal calf serum that was previously treated with AG 1-X8 resin and charcoal (AXC serum).

Cell growth inhibition studies by MTT assay: Cell viability study was measured with the conventional MTT-reduction assay, as described previously with a slight modification11. Cells were seeded in a 96-well plate at the density of 5×103 cells/well. The cells were allowed to attach and grow in a 96-well plate for 24 hrs, in 200 µl of D-MEM with 10% FBS and various concentrations of purified compound viz., 100 µg/ml, 50 µg/ml, 10 µg/ml, 1 µg/ml (minimum 3 wells were seeded with each concentration) and incubated for 48 hrs. The cell media was removed and replaced with fresh medium. After treatment, the cells were incubated with MTT (10 µl, 5 mg/ml) at 370°C for 4 hrs and then with DMSO at room temperature for 1 hour. Two wells per plate without cells served as blank. All experiments were repeated three times in triplicates. The plates were read at 495 nm on a scanning multi well spectrophotometer.

\[
\text{% cell viability} = \left( \frac{\text{Absorbance of plant extract treated sample}}{\text{Absorbance of control}} \right) \times 100
\]

RESULTS AND DISCUSSION

Extraction and purification of metabolite: About 5 g of crude extract was loaded on to silica gel (100-200 mesh) column chromatography and fractionated using chloroform and methanol as eluting solvent system in
gradient passion. Altogether, seven fractions were obtained and the fractions with Rf values 0.876, 0.765, 0.634 and 0.500 were recognized to possess α-amylase inhibitory activity. Among the fractions, C-1 showed remarkable α-amylase inhibitory activity and the fractions 2, 3 and 4 did not show any significant activity (Table 1). Hence, the fraction C-1 was subjected to high resolution silica gel column chromatography using 230-400 mesh silica gel and the same solvent system chloroform/methanol. There were two fractions obtained with different Rf in TLC. The fraction with significant antidiabetic activity was selected for further structural elucidation using various spectral analyses.

Physical properties: An orange yellow oily crystal compound with no UV absorbing band on TLC with the Rf value of 0.88 (9:1, chloroform: methanol) was purified from G. hirsuta (Fig.1). This antidiabetic compound was fully soluble in organic solvents such as chloroform, hexane, methanol and DMSO but sparingly in water. The melting point of the compound was found to be 68-690C.

MTT assay: The MTT results were used to determine the concentration, at which maximum percentage of cell viability was obtained. It is evident from the results that, as the compound concentration increases from 1to100 g/ml, there is a decrease in the percentage viability of 3T3 cell lines from 86.08 to 76.75% when compared with solvent control (90.85%) (Fig. 2) and for L6 cell lines the cell viability percentage was found to range from 89.61% to 78.62% with solvent control (90.45%) (Fig.3). No significant reduction observed in the cell viability of 3T3 and L6 cell lines at any concentration up to 1 g/ml.

Spectral studies: The UV absorption spectrum of the purified compound showed the absorption maximum at 221 nm (near UV) revealing the presence of double bond. Since it is a coloured compound with conjugation, absorption was found in the visible region at 528 nm.

The IR spectrum of the purified compound displayed a strong aliphatic C-H stretching band at umax 2924-2853 cm-1. Two strong bands at umax 1711 and at umax 1641 cm-1 indicates the presence of C=O and alkyl C=C stretch respectively. There is a band at umax 1020.43cm-1 indicating C=C-H bending (Fig. 5).

The Q-TOF spectrum of the purified compound of G. hirsuta showed a molecular ion peak at m/z 220 (100%), indicating the molecular weight of the purified compound (Fig. 6).

The 1H NMR shift of the purified compound showed the olefinic proton as multiplet δ 5.02-5.13 and other CH2 proton appeared as multiplet at δ 0.58-2.09 (Fig. 7).

The 13C NMR spectrum showed the aliphatic carbons appearing at 16.01, 17.68, 22.71, 23.44, 25.69, 26.43, 26.71, 29.72, 31.95, 32.24 and 39.75 ppm. The carbonyl carbon appeared at δ 173.57, the olefinic carbon appeared at δ 124.45 and 134.90 (Fig. 8).

Structural elucidation: The mass fragmentation search in the Mass Bank and molecular weight search between 150 and 300 in the Novel Antibiotic database, PubChem, NIST and SDBS databases revealed a similar compound but with a change in the position of –CH2 group. Based on the physico–chemical properties, the purified compound was identified as (4Z, 12Z)-cyclopentadeca-4, 12-diene and its structure is depicted in Fig. 9. Its molecular formula is C15H24O. Its calculated and exact molecular weight is 220 g/mol. Reports suggest that polyphenolic compounds, especially flavonoids, are among the classes of compounds that have received the most attention with regard to their antidiabetic properties.

CONCLUSION

The results of the current study have revealed (4Z, 12Z)-cyclopentadeca-4, 12-diene compound with potent antidiabetic efficacy. MTT assay studies revealed that the percentage of cell viability was found to decrease as the concentration of this compound increases. The extension of the work through docking studies using this compound is on progress. Further the antioxidant and anti proliferative studies for this compound can be studied.

ACKNOWLEDGEMENT

The authors are sincerely thankful to Dr. M. Krishnaraj, Jayagen Biologics, Chennai for his extensive support in carrying out the characterization of the compound.

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