**Original Article**

**α-Amylase Inhibition, Antioxidant Activity and Phytochemical Analysis of *Calotropis gigantea* (L.) Dryand**

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**ABSTRACT**

Introduction: Antioxidant and α-Amylase inhibitory activity of methanolic extract of *Calotropis gigantea* (L.) Dryand leaves were evaluated.

Methods: The antioxidant activity was evaluated by DPPH assay. The extract was fractionated in Silica gel loaded column chromatography (CC). All fractions were evaluated for their purity by TLC. Out of 11 fractions from CC, one fraction was analyzed by Gas Chromatography-Mass Spectrometry (GC-MS).

Results: The antioxidant activity of methanolic extract was found satisfactory (IC₅₀ 268.80 µg/ml) as compared with ascorbic acid (141.82 µg/ml). TLC of a fractions showed a compound at Rf value at 0.45 in toluene: chloroform: methanol with mobile phase ratio 7:2:1 respectively.

Conclusion: Total 17 compounds were identified by GC-MS of ethyl acetate fraction and 5-hydroxyl methyl furfural was major furan compound (59.49%). α-Amylase inhibitory activity of the same fraction showed IC₅₀ value of 0.94 mg/ml. The Nepalese originated *C. gigentea* (L.) Dryand possesses antioxidant and α-Amylase inhibitory property.

**Keywords:** *Calotropis gigantea* (L.) Dryand, α-Amylase inhibitory, Antioxidant activity, Fractionation, GC-MS, 5-Hydroxyl methyl furfural

**INTRODUCTION**

Herbal medicine and their drugs are gaining popularity, since they are derived from medicinal plants, minerals and organic matter.¹ A number of medicinal plants, traditionally used for over 1000 years named Rasayana are present in herbal preparations of Ayurvedic health care systems.² *Calotropis gigantea* (L.) Dryand is a weed and is common to Bangladesh, Burma, China, India, Indonesia, Malaysia, Pakistan, Philippines, Thailand, Nepal and Sri Lanka. *C. gigantea* (L.) Dryand flowers are extensively used by Ayurvedic physicians for treatment of disorders such as diabetes mellitus, bronchial asthma, rheumatoid arthritis, and nervous disorders.³ The flowers are reported to possess analgesic activity,⁴ antimicrobial and cytotoxic activity.⁵ Leaves and aerial parts of the plant are reported for anti-diarrhoeal activity,⁶ anti-candida activity,⁷ antibacterial activity,⁸ antioxidant activity,⁹ and anti-diabetic activity.¹⁰ *C. gigantea* (L.) Dryand is reported to possess alkaloids, cyanogenic, glycosides, phenolics, tannins, cardenolides,¹¹,¹² flavonoids,¹³ terpenes and sterols,¹⁴ as major phytochemical groups.

To promote the use of medicinal plants as potential sources of bioactive compounds, it is important to find out their phytochemicals thoroughly by isolation, analysis and evaluation of their compounds. DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts. The α-Amylase inhibition is considered a strategy for the treatment of disorders in carbohydrate uptake, such as diabetes, obesity, dental caries and periodontal diseases. Taking this into consideration, α-Amylase inhibition, antioxidant activity and phytochemical analysis of Nepalese originated *C. gigantea* (L.) Dryand were evaluated.

**METHODS**

**Chemicals**

Methanol, n-hexane, chloroform, ethyl acetate used were purchased from Changshu Hongsheng Fine Chemical Co. Ltd, China. DPPH reagent was purchased from Sigma-Aldrich chemicals Pvt. Ltd., India. α-Amylase from malt was purchased from Sigma-Aldrich.

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from HIMEDIA chemicals, India. All the chemicals used in the study are of analytical grade.

**Collection and Extraction**
The leaves of *C. gigantea* (L.) Dryand were collected from Panchkhal, Kavrepalanchowk district of Nepal during May 2019 from 850 m altitude. The plant was identified by Botanist Tirtha Maiya Shrestha, visiting faculty of Department of Pharmacy, Kathmandu University, Nepal. A voucher specimen (DOP-H-21) which was deposited in Department of Pharmacy, Kathmandu University, Nepal. They were washed, dried for two weeks under the shade, powdered, sieved by 40 mesh sieve, and extracted with methanol by using maceration method. The methanolic extract was further fractionated into n-hexane, chloroform, ethyl acetate solvents, then evaporated in rotary evaporator at 40 °C water bath temperature.

**Phytochemical Screening**
Phytochemical analysis of methanolic extract was screened for alkaloid, glycoside, tannins, flavonoid, coumarin and reducing sugar by following the standard guideline.\(^{15}\)

**Thin Layer Chromatography**
The TLC was performed on ready made Silica gel TLC plates as previously described.\(^{16}\) The plates were dried in hot air oven at 110°C for 30 minutes, stored at room temperature of 25°C and then immediately used for analysis. Samples of all fractions were prepared by diluting the crude extracts (1:10 W/V) and then applied usually 10μl volumes to the origins of a TLC plate 2 cm above its bottom with the help of capillary tubes and run for 20 minutes at room temperature in TLC chamber. After series of experimentation to select the suitable combination of mobile phase, the mobile phase used was toulene: chloroform and methanol in 7:2:1 respectively for ethyl acetate column chromatography fraction to obtain the single spot. Exposure of TLC plates was done by UV light at 254 nm. Different bands were observed and corresponding Retention factor (Rf) value was determined as:

\[ Rf = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}} \]

**Gas Chromatography-Mass Spectroscopy**
The selected column chromatography fraction (combined Fr6 and Fr7) of ethyl acetate extract originally from alcoholic extract was analyzed by Shimadzu GCMS-QP-2010 plus using capillary column (25 m × 0.25 mm i.d. x 0.25 mm thick film). The GCMS was programmed as following; 50°C during 5 minutes, increased to 200°C at 10°C/min. Helium was used as the carrier gas with a constant flow rate of 1.2 ml min-1 and injection volume of 1.0 μL of a solution. Injector temperature was 180°C and pressure 36.9 kPa. The chemical constituents were identified by comparison of mass spectra and retention indices (RI) with those in the literature. The libraries WILEY and NIST were also used to assist in the identification of compounds.

**Antioxidant Activity**
The antioxidant activity of the extracts was determined using the DPPH free radical scavenging assay previously described with some modifications.\(^{17}\) Briefly, stock solution of 100μM DPPH in methanol was prepared. The methanolic extract was prepared in 25, 50, 100, 200, 400μg/ml concentrations in methanol. Similarly, reference sample of ascorbic Ascorbic acid as positive control without sample. 2.0 ml of 100μM DPPH was added to 2.0 ml of each extracts at different concentration and kept in dark. Similarly, 2.0 ml of 100μM DPPH was mixed with 2.0 ml of methanol and ascorbic acid and kept in dark for 30 minutes in incubator at 37°C. The absorbance was measured at 517 nm by UV spectrophotometer after 30 minutes and % scavenging was calculated by the following equation:

\[ \text{Percentage scavenging} = \left( \frac{\text{Ao} - \text{AT}}{\text{Ao}} \right) \times 100\% \]

Where, \( \text{Ao} = \text{Absorbance of DPPH solution and AT = Absorbance of test or reference sample.} \)

The % scavenging was then plotted against concentration and regression equation was obtained to calculate IC50 (micro molar concentration required to inhibit DPPH radical formation by 50%) values.

**α-Amylase Activity**
The α-amylase inhibition assay was carried out using 3,5-dinitrosalicylic acid (DNS reagent), following the procedure.\(^{18}\) Slight modifications have been made for ease of use. 20mM phosphate buffer (pH 6.9) containing 6.7mM NaCl (hereafter buffer) and 96 mM DNS reagent. Solutions of 20, 40, 60, 80 and 100 ppm of acarbose I.P. and 1.5, 10, 15, 20 and 40 ppm concentrations of ethyl acetate extract were prepared in buffer. All the reagents were pre-incubated at 35°C. A total of 0.5ml of each inhibitor was placed in individual test tubes and 0.5ml of buffer containing-α-amylase (Himedia RM638) solution (1 mg/
ml) was added to it. To these solutions, 0.5ml each of 1% starch solution in buffer was added as quickly as possible. Individual volumes of amylase solution and inhibitor were replaced with buffer in sample blank and maximum enzyme activity (control) respectively. All the reaction mixtures were incubated for exactly 3 minutes at 35°C. The reaction was terminated by addition of 0.5ml DNS reagent. The tubes were then incubated in boiling water bath for 15 min and cooled to room temperature. The reaction mixture was diluted with 4.5ml double distilled water and the absorbance was measured at 540 nm. The α-amylase inhibitory activity of the extract and acarbose was calculated using the formula: % Inhibition = [(Abs Control - Abs Sample)/ Abs Control] x 100
The concentrations of individual extracts resulting in 50% inhibition of α-Amylase activity (IC₅₀) were determined graphically.

Statistical Analysis
Data were analyzed by SPSS 16.0.0. Equations to estimate IC₅₀ values were obtained by linear method.

RESULTS AND DISCUSSION

Phytochemical Screening
Preliminary phytochemical screening of the C. gigantea (L.) Dryand showed that the extract (yield 12.5%) consists of glycosides, alkaloids and flavonoids as a major class of secondary metabolites. Similar result was also obtained in the previous findings on same plant from different geographical origin.¹⁹,²⁰

Thin Layer Chromatography
In this work, thin layer chromatography (TLC) known as the easiest, cheapest, cost-effective, and easy-to-operate planar chromatographic techniques was adopted to recognize the secondary metabolites present in these botanicals. The principle involves separation of organic compounds on thin layers of adsorbents coated glass by their retention factor (Rf). This method provides a clue about the polarity of secondary metabolites to determine the best solvent for the separation of bioactive compounds in column chromatography. Here, the TLC profiling of ethyl acetate extract indicated the separate one spot in upon exposure. Though the all fractions were evaluated in TLC by selecting mobile phases randomly but a compound with high density was detected having Rf value at 0.45 in toluene: chloroform: methanol mobile phase with the ratio 7:2:1 respectively. One drop of trimethyl amine was added in mobile phase for visualization of spot clearly.

Figure 1: TLC of ethyl acetate fraction from Methanolic extract of C. gigantea (L.) Dryand

Column Chromatography
The huge number of medicinal plants have been investigated worldwide to isolate the bioactive compounds. Our result was found to be interesting because of the naturally occurring new compounds with significant medicinal values were detected after the column chromatography of ethyl acetate fraction, which was ultimately fractionated into eleven fractions. Among the fractions, some of the fractions may deserve the property of bioactivity which is found completely depending on nature of chemical constituent obtained and targeted medicinal property.

Table 1: Fractions and mobile phase used for column chromatography of ethyl acetate extract of C. gigantea (L.) Dryand.

| Fractions | Solvent Ratio(Toluene :Chloroform :Methanol) |
|-----------|---------------------------------------------|
| Fr1       | 7:2:1                                       |
| Fr2       | 5.5:2:2.5                                   |
| Fr3       | 5:2:3                                       |
| Fr4       | 4.5:2:3.5                                   |
| Fr5       | 3.5:2:5.4                                   |
| Fr6       | 3:2.5:4.5                                   |
| Fr7       | 2.75:2.75:4.25                              |
| Fr8       | 2.5:3:4.5                                   |
| Fr9       | 2:3:5                                       |
| Fr10      | 1:3:6                                       |
| Fr11      | 0:3:7                                       |

Gas Chromatography Mass Spectrometry (GC-MS)
Series of TLC showed that fraction Fr6 and Fr7 were similar in compositions. So we mixed them together and finally forwarded for the GC-MS. The result of GC-MS showed that the fraction contained 17 compounds (Table 2). Among which, furan compound, 5-hydroxyl methyl furfural was a most dominant compound with (59.45%). This compound was previously also isolated in the plant sample and other phytochemical work.²¹
Figure 2: GC-MS Chromatogram of C. gigantea (L.) Dryand fraction obtained from column chromatography of ethyl acetate extract.

Table 2: Compounds obtained from GC-MS of column chromatography fractionated ethyl acetate extract of C. gigantea (L.) Dryand.

| S.N. | Retention Time | % Area | Compounds                                                                 |
|------|----------------|--------|---------------------------------------------------------------------------|
| 1    | 9.44           | 5.21   | Furfural                                                                  |
| 2    | 14.68          | 2.88   | 5-Methyl furfural                                                          |
| 3    | 18.47          | 2.09   | Phenyl acetaldehyde                                                        |
| 4    | 20.33          | 1.64   | N-Methyl-2-furohydroxamic acid                                             |
| 5    | 20.98          | 3.52   | 1-Undecyne                                                                |
| 6    | 21.55          | 3.41   | 1-Methyl-4-(1-methylethyl)-Cyclohexanol                                    |
| 7    | 21.86          | 1.7    | 3-Methyl-4-(phenylthio)-2-prop-2-enyl-2,5-dihydrothiophene-1,1-dioxide     |
| 8    | 23.24          | 7.89   | 2-Propyl-tetrahydropryan-3-ol                                              |
| 9    | 23.68          | 1.36   | Levomenthol                                                               |
| 10   | 23.87          | 1.07   | 5-Mercaptopentazole                                                       |
| 11   | 24.84          | 1.17   | 3-Hydroxy-1-methylpyridinium hydroxide                                     |
| 12   | 24.93          | 1.87   | Methyl salicylate                                                         |
| 13   | 27.31          | 59.39  | Hydroxy methyl furfural                                                    |
| 14   | 38.42          | 2.87   | Methyl 4-azido-4-deoxy- .beta.-l-ylopyranoside                            |
| 15   | 42.31          | 1.68   | 1,5-Anhydro-d-mannitol                                                     |
| 16   | 46.09          | 1.19   | 1,3-Di(cyclohexyl)butane                                                  |
| 17   | 47.13          | 1.07   | Pentadecanal                                                              |

Antioxidant Activity
The antioxidant activity of the methanolic extracts was determined by DPPH method and the results are presented in Table 3. Our experimental results showed that the average antioxidant activity was found in extract in comparison to standard ascorbic acid (IC50 141.82 µg/ml). Similar type of result was also obtained in previous findings.22

Table 3: Antioxidant activity of methanolic extract of C. gigantea (L.) Dryand

| Extract concentration (mcg/ml) | % Scavenging | IC₅₀ (mcg/ml) |
|-------------------------------|--------------|--------------|
| 25                            | 9.6          |              |
| 50                            | 19.03        |              |
| 100                           | 28.46        | 268.80       |
| 200                           | 39.8         |              |
| 400                           | 68.65        |              |

α-Amylase Inhibitory Activity
The inhibition of pancreatic α-Amylase is one of the therapeutic targets for delaying oligosaccharide digestion to absorbable monosaccharides in the intestinal brush border, resulting in reduced postprandial hyperglycemia23. The α-amylase inhibitory activity of the fractionated ethyl acetate extract was determined and the results are presented in Table 4. In our experimental results α-Amylase activity was found at average level in extract in comparison to standard acarbose (IC50 184.56 µg/ml). It was found that extracts could serve as a source of antidiabetic agents which may act through the inhibition of carbohydrate hydrolyzing enzymes, α-Amylase24.

Table 4: α-Amylase inhibitory property of ethyl acetate fraction of C. gigantea (L.) Dryand

| Extract concentration (mcg/ml) | % Inhibition | IC₅₀ (mcg/ml) |
|-------------------------------|--------------|--------------|
| 1                            | 10.14        |              |
| 5                            | 20.91        |              |
| 10                           | 27.14        |              |
| 15                           | 29.91        | 24.1107      |
| 20                           | 61.08        |              |
| 40                           | 68.42        |              |

Plentoria of research showed that ample numbers of pharmacological activities of C. gigantea (L.) Dryand were studied for the phytochemistry and bioactivities. C. gigantea (L.) Dryand has demonstrated its analgesic activity, antimicrobial activity, antioxidant activity, anti-pyretic activities.1-10 Alkaloids, glycosides, flavonoids were detected as major class of metabolites in our study is also similar findings with the previous works.11-13,25,26 Wide range of medicinal properties of the plant leaves are mainly due to the presence of such metabolites.11-15

CONCLUSION
Our result suggests that fractions of the ethyl acetate extract of C. gigantea (L.) Dryand, enriched with flavonoids, appear as a promising source of natural antioxidants and α-amylase inhibition property. Hydroxymethyl furfural was a major constituent found in the ethyl acetate fraction of the leaf extract, which could be used for the further targeted pharmacological activities.

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