JHAS 2020; 10(1):77-81
doi:https://doi.org/10.37107/jhas.143

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

Rajendra Gyawali,[1] Bijay Bhattarai,[1] Susan Bajracharya,[1] Surakshya Bhandari,[1] Puja Bhetwal,[1] Kanchan Bogati,[1] Sahana Shrestha,[1] Ashwinee Kumar Shrestha,[1] Shardul Neupane,[2] Rajendra Joshi,[3] Prem Narayan Paudel[3]

[1]Department of Pharmacy, Kathmandu University, Dhulikhel, Nepal,[2] Department of Biotechnology, Kathmandu University, Dhulikhel, Nepal,[3] Department of Chemical Science and Engineering, Kathmandu University, Dhulikhel, Nepal.

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 (IC_{50}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 IC_{50} value of 0.94 mg/ml. The Nepalese originated *C. gigentea* (L.) Dryand possesses antioxidant and α-Amylase inhibitory property.

Keywords: *Calotropis gigentea (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 antidiabetic 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

Correspondence: Rajendra Gyawali, Department of Pharmacy, Kathmandu University Dhulikhel, Kavre, GPO Box. 6250, Nepal, email : ragyawali@gmail.com

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 0C 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: methanol and optimized the ratio of 7:2:1 respectively for ethyl acetate fraction to obtain the single spot. Exposure of TLC plates was done by UV light at 254nm. Different bands were observed and corresponding Retention factor (Rf) value was determined as:-

Rf = Distance travelled by the solute / Distance travelled by the solvent.

Column Chromatography

Column chromatography of ethyl acetate fraction was performed in 120 cm long glass column by wet packing of Silica gel of mesh size 60 making slurry with water. Gradient elution of mobile phases toulene, chloroform and methanol were used in pure and mixture form (Table 1). The plant extract was mixed with Silica gel and loaded into the column. The column was eluted repeatedly with solvent without disturbing the loaded extract on column.

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 GC-MS was programmed as following; 50ºC 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:

Percentage scavenging = (Ao−AT)/ Ao x 100%.

Where, Ao = 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 35ºC. A total of 0.5ml of each inhibitor was placed in individual test tubes and 0.5ml of buffer containing-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 4.5 ml 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 (IC_{50}) were determined graphically.

Statistical Analysis

Data were analyzed by SPSS 16.0.0. Equations to estimate IC_{50} 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.

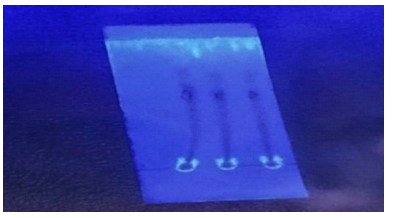

Figure 1: TLC of ethyl acetate fraction from Methanolic extract of *C. gigantea* (L.) Dryand

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[21] and other phytochemical work[19].

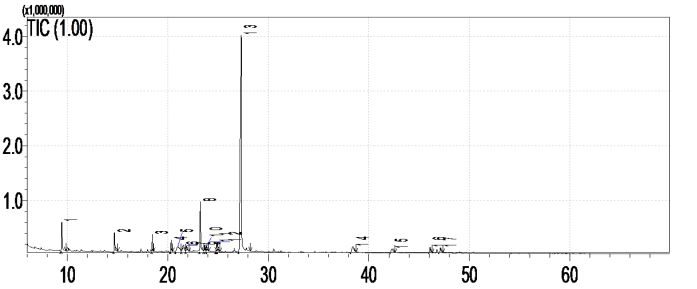

Figure 2: GC-MS Chromatogram of *C. gigantea* (L.) Dryand fraction obtained from column chromatography of ethyl acetate extract.

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-tetrahydropyran-3-ol
9	23.68	1.36	Levomenthol
10	23.87	1.07	5-Mercaptotetrazole
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	IC_{50} (mcg/ml)
25	9.6	268.80
50	19.03	
100	28.46	
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 hyperglycemia[23]. 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-Amylase[24].

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

Extract concentration (mcg/ml)	% Inhibition	IC_{50} (mcg/ml)
1	10.14	24.1107
5	20.91	
10	27.14	
15	29.91	
20	61.08	
40	68.42	

Plethora 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.[3-10]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.

ACKNOWLEDGMENT

The authors are very much thankful to institutional research grant support of University Grant Commission (UGC) Nepal for supporting this research work. Our gratitude is also extended to the Department of Pharmacy, Kathmandu University, Nepal.

REFERENCES

1. Grover JK, Yadav S, Vats V, Medicinal plants of India with antidiabetic potential. J. Ethnopharmacol. 2002;81:81–100.
2. Scartezzini P, Sproni E, Review on some plants of Indian traditional medicine with antioxidant activity. J. Ethnopharmacol. 2000;71:23–43.
3. Meena, AK & Yadav, A. & Meda MR, Mruthyumjaya. Ayurvedic uses and pharmacological activities of Calotropis procera Linn. Asian J Tradit Med. 2011, 6: 45-53.
4. Pathak AK, Argal A, Analgesic activity of Calotropis gigantea flower. Fitoterapia 2007;78(1):40-42.
5. Habib MR, Karim MR, Antimicrobial and Cytotoxic Activity of Di-(2-ethylhexyl) Phthalate and Anhydrosophoradiol-3-acetate Isolated from Calotropis gigantea (Linn.) Flower. Mycobiology 2009; 37(1):31-36.
6. Chitme HR, Chandra R, Kaushik S, Studies on anti-diarrhoeal activity of Calotropis gigantea R. Br. in experimental animals. J Pharm Pharmaceut Sci , 2004;7(1):70-75.
7. Kumar G, Karthik L, Bhaskara Rao KV, In vitro anti-Candida activity of Calotropis gigantea against clinical isolates of Candida. Journal of Pharmacy Research 2010;3(3):539-542.
8. Kumar G, Karthik L, Bhaskara Rao KV, Antibacterial activity of aqueous extract of Calotropis gigantea leaves – an in vitro study. International Journal of Pharmaceutical Sciences Review and Research 2010;4(2):141-144.
9. Singh N, Jain NK, Kannojia P, Garud N, Pathak AK, Mehta SC, In vitro antioxidant activity of Calotropis gigantea hydroalcohlic leaves extract. Der Pharmacia Lettre 2010;2(3):95-100.
10. Manivannan R, Shopna R, Antidiabetic activity of Calotropis gigantea white flower extracts in alloxan induced diabetic rats, Journal of Drug Delivery and Therapeutics. 2017; 7(3):106-111
11. Lhinhatrakool T, Sutthivaiyakit S, 19-Norand 18, 20-Epoxy-cardenolides from the leaves of Calotropis gigantea (L.) Dryand. J. Nat. Prod. 2006;69(8):1249-1251.
12. Seeka C, Sutthivaiyakit S, Cytotoxic cardenolides from the leaves of Calotropis gigantea (L.) Dryand. Chem. Pharm. Bull. 2010;58(5):725-728.
13. Sen S, Sahu NP, Mahato SB, Flavonol glycosides from Calotropis gigantea. Phytochemistry 1992;31(8):2919-2921.
14. Gupta J, Ali M, Rare chemical constituents from Calotropis gigantea (L.) Dryand roots. Indian J. Pharm. Sci. 2000;62(1):29-32.
15. Trease GE, Evans WC. Pharmacognosy. 15th Ed. London: Saunders Publishers; 2002.
16. Biradar RS, Rachetti DB (2013). Extraction of some secondary metabolites & thin layer chromatography from different parts of Centella asiatica L. American Journal of Life Sciences. 1(6): 243-247.
17. Nithianantham K, Shyamala M, Chen Y, Latha LY, Jothy SL, Sasidharan S. Hepatoprotective Potential of *Clitoria ternatea* Leaf Extract Against Paracetamol Induced Damage in Mice. Molecules 2011;16:10134–10145.
18. Bernfeld, P, Amylases: alpha and beta methods. Enzymol. 1955;1: 149-158.
19. Thakur S, Das P, Itoh T. Kazunori Imai, Taro M. Latex extractable of Calotropis gigantea (L.) Dryand. Phytochemistry 1984;9:2085- 2087.
20. Sen S, Sahu NP, Mahato SB. Flavonol glycosides from Calotropis gigantea. Phytochemistry 1992;31(8):2919-2921.
21. Nguyen KDH, Dang PH, Nguyen HX, Nguyen MTT, Awale S, Nguyen NT. Phytochemical and cytotoxic studies on the leaves of Calotropis gigantea. Bioorganic Med Chem Lett. 2017;27(13):2902–2906.
22. Rathod NR, Raghuveer I, Chitme HR, Chandra R. Free Radical Scavenging Activity of Calotropis gigantea on Streptozotocin-Induced Diabetic Rats. Indian J Pharm Sci. 2009;71(6):615–621.
23. Sim L, Jayakanthan K, Mohan S, Nasi R, Johnston BD, Pinto BM, Rose DR. New glucosidase inhibitors from an Ayurvedic herbal treatment for type 2 diabetes: structures and inhibition of human intestinal maltase-glucoamylase with compounds from Salacia reticulata, Biochemistry, 2010, 49(3), 443–451.
24. Kazeem MI, Mayaki AM, Ogungbe BF, Ojekale AB. In-vitro Studies on Calotropis procera Leaf Extracts as Inhibitors of Key Enzymes Linked to Diabetes Mellitus. Iran J Pharm Res. 2016;15(Suppl):37–44.
25. Habib MR, Nikkon F, Rahman M, Haque ME, Karim MR, Isolation of Stigmasterol and β-Sitosterol from methanolic extract of root bark of Calotropis gigantea (Linn). Pak. J. Biol. Sci. 2007;10(22):4174-4176.
26. Pal G, Sinha NK, Isolation, crystallization, and properties of calotropins DI and DII from Calotropis gigantea (L.) Dryand. Archives of Biochemistry and Biophysics 1980;202(2):321-329.