October - November 2017; 6(6): 2800-2805

International Journal of Research and Development in Pharmacy & Life Science
An International open access peer reviewed journal
ISSN (P): 2393-932X, ISSN (E): 2278-0238
Journal homepage: http://ijrdpl.com

Original Article

Phytochemical analysis, exploration of antidiabetic and antioxidant potential of *Anthocephalus cadamba* (Roxb.)

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Keywords: Antidiabetic, Antioxidant, methanolic extract, cadambine, *Anthocephalus cadamba*

Article Information:

Received: August 07, 2017;
Revised: August 31, 2017;
Accepted: September 28, 2017

Available online on:
15.10.2017@http://ijrdpl.com

http://dx.doi.org/10.21276/IJRDPL.2278-0238.2017.6(6).2800-2805

ABSTRACT: The most common disease nowadays is diabetes. In a fast-changing world, many means to treat diabetes naturally are explored by experts and clinicians today. Continuing use of insulin and other oral hypoglycemic agent creates undesirable side-effects, consequentially, unrestrained increase in blood sugar as well as complications associated with heart diseases; diabetics may have to face. To evade such glitches, herbal medications has superior advantages. As an alternative of using allopathic formulations, it is thus, gainful to use ayurvedic preparations for healthier regulation of diabetes mellitus. Current research was thus taken on to explore the anti-diabetic and antioxidant activities of crude extracts and pure compound obtained from the bark of *Anthocephalus cadamba*. The methanolic extract exhibited promising antidiabetic and antioxidant activity. Grounded on these results, the methanolic extract was fractionated on a silica gel column chromatography in a bioassay-led fractionation resulting in one known isolate, cadambine showing potent anti-diabetic activity, more than that of the positive control, glibenclamide. The results indicated that *Anthocephalus cadamba* methanolic extract (ACME) and the isolated compound (Cadambine) are potential natural agents to control diabetes. Alloxan significantly induced hyperglycemia; Oral administration of test samples for 14 days caused a significant decrease in blood glucose levels. The possible mechanism by which ACME mediated its antidiabetic effect could be by potentiation of pancreatic secretion of insulin from existing β-cells of islets in the extract treated animals.

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INTRODUCTION

Diabetes mellitus (DM) comprises of a collection of syndromes categorized by hyperglycemia, transformed protein, carbohydrate and lipid metabolism and an amplified risk of snags from vascular disease [1]. In diabetes, hyperglycemia produces reactive oxygen species, which sequentially causes lipid peroxidation and membrane mutilation, produces secondary complications in DM (kidney, eye, blood vessel & nerve injury [2]. Many herbal formulations are recommended nowadays for the treatment of diabetes as the conventional oral hypoglycemic agents and insulin possess certain drawbacks of their own [3].

Many remedial plants have been explored and their therapeutic efficacy has been widely exploited in complex disorders such as diabetes [4].

Anthocephalus cadamba (*A. cadamba*) (Roxb.) Miq. (Rubiaceae) is known as wild cinchona and popular in India as "Kadamb". Many portions of this plant have conventionally been used as an antidiuretic, in the treatment of fever, anemia and tumor, and for the enhancement of semen quality [5, 6]. The bitter and pungent bark of the plant is used to treat uterine complications, blood diseases, leprosy and dysentery as an ayurvedic formulation. Leaf decoction is used as a gargle in case of stomatitis.

The crude extracts from *A. cadamba* have been shown to possess biological activities viz., anti-inflammatory [7] anti-hepatotoxic activities [8], analgesic activity [9], antimicrobial and anthelmintic activities [10] and hypoglycemic activity [11]. The plant, *Anthocephalus cadamba* has been reported to contain glycans, saponins, glycoproteins, peptides, amino acids, terpenoids, alkaloids and flavonoids as their chief phytochemical constituents which has been credited for hypoglycemic activity [12-14]. Thus, the major objective of this study was to isolate the active constituent from the plant and to assess the anti-diabetic potential of *Anthocephalus cadamba in vivo*.

MATERIALS AND METHODS

Chemicals

Alloxan was procured from CDH, New Delhi and glimepiride was received from Ross Robinz Biotech, Solan (H.P) as gift sample. All other chemicals and reagents used were of analytical grade.

Plant material

The fresh stem bark of *Anthocephalus cadamba* was collected during the month of September 2015, from village Gandouli Konch Distt- Jalaun (U.P.). The plant material was taxonomically identified and authenticated by Dr. Gaurav Nigam, Botany Department, Bundelkhand University, Jhansi (Herbarium and Museum Division with ref. no. BU/BOT /375/24-01-2015).

Preparation of plant extract

Extraction of plant

The powder of the stem bark of *Anthocephalus cadamba* was successively extracted with petroleum ether, chloroform, methanol, and water, respectively. Portion of each extract was dried under vacuum and assayed for antidiabetic and antioxidant activities. Results revealed activity in the methanolic extract, a large-scale of plant sample (3.0 kg) were extracted with methanol at room temperature for 24 h. The extract was evaporated to dryness under reduced pressure in a rotary evaporator to yield the crude extract (332.0 g).

This extract was subjected to column chromatography using silica gel and successively eluted with petroleum ether/chloroform/methanol gradient composition from low polarity index to high. The elution process was monitored with thin layer chromatography (TLC).

Elutes showing same Rf values were combined and proceeded to preparative thin layer chromatography (pTLC) to obtain single components. Recrystallization of the isolate from methanol fraction, with alcohol led to compound Cadambine (1,650.0 mg). The structures of Cadambine were established with the help of physicochemical and spectral data (nuclear magnetic resonance and mass spectra) by comparison with literature values (**fig. 1**) [15].

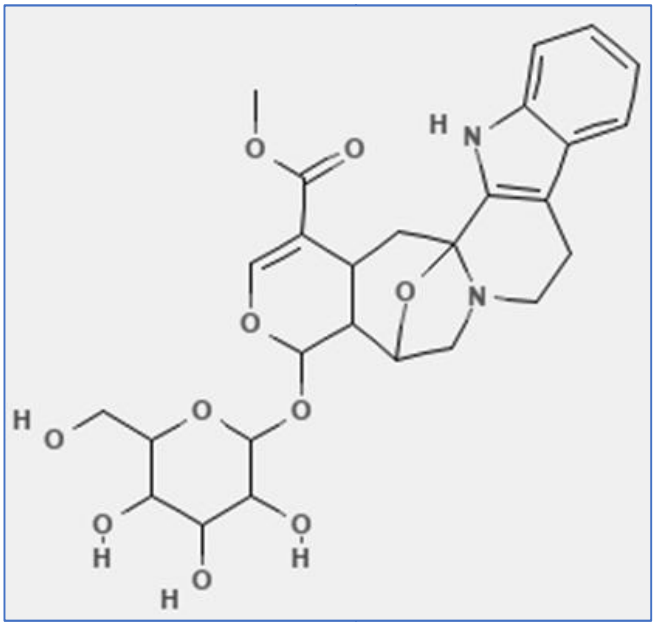

Fig. 1: Structure of Cadambine

Anti-diabetic studies

Experimental Animals

The adult male albino rats of weight 180-240 gm were selected for the study. All animals were procured from disease free animal house, Institute of Pharmacy, Bundelkhand University, Jhansi with Institutional ethical number- (IAEC No. BU/Pharm/16/01A).

The animals were housed in polypropylene cages, 5 per cage with free access to standard laboratory diet and water *ad libitum*. The rats were maintained under standard laboratory conditions at 25±2^{o}C, relative humidity 50±15% and normal photo period (12 h dark/ 12h light) were used for experiment.

Chemicals

Alloxan (purchased from CDH, New Delhi) was used for the induction of diabetes and the standard drug i.e. glibenclamide was received from Sun Pharmaceutical Industries, J & K.

Preparation of test sample

For *in vivo* bioassay, the test samples were suspended in 0.5% carboxymethylcellulose (CMC) in distilled water at a concentration of 0.2 and 0.4 g/kg for crude methanol extract, 10 mg/kg for pure compound (Cadambine) prior to oral administration (5 ml/kg) to the experimental animals [16] and stored at 20^{o}C until further required.

Glibenclamide at a concentration of 10 mg/kg was used as a standard drug [16] and animals in control group received only the 0.5% CMC (5 ml/kg). All the drugs were administered four times on the first day (1, 2, 4, and 6 h) and single time on 2nd, 4th, 7th, and 14th day.

Effect of methanolic extract on alloxan induced diabetic rats

Induction of experimental diabetes

Diabetes mellitus was induced by administering intraperitoneal injection of alloxan monohydrate 120 mg/kg [17] to the overnight fasted rats. Five days after administration of alloxan, fasting blood glucose of 300 to 450 mg/dl were included in the study.

Sample collection

Blood sample were collected from tail nipping and glucose level was determined by an automatic electronic glucometer (Accuchek comfort).

Evaluation of Anthocephalus cadamba methanolic extract (ACME) & isolated compound on alloxan induced diabetic rats

Diabetes in experimental animals was induced by intraperitoneal (i.p.) injection of alloxan at a dose of 150 mg/kg in normal saline solution (1 ml/kg) to overnight fasted rat. Fasting blood glucose levels were measured after 5 days, animals with blood concentration above 200 mg/dl was considered to be diabetic. The animals were then randomly assigned into six groups of six animals each and received the following treatment: Group I: Normal control +distilled water, Group II: Diabetic control + distilled water, Group III: Diabetic + ACME (0.2 g/kg), Group IV: Diabetic + ACME (0.4 g/kg), and Group V: Diabetic + cadambine (10 mg/kg), VI: Diabetic + Glibenclamide (10 mg /kg) The freshly prepared solutions were orally administered daily for 14 days. Body weights were measured weekly on overnight fasted animals. At the end of the experimental period, the animals were fasted overnight and blood was collected for various biochemical estimations.

Procedure

Biochemical analysis

Serum glucose analysis was done by GOD-POD method using Glucometer, Accucheck-COMFORT® (Roche-Diagnostics). Other serum estimation was done spectrophotometrically using standard kits available which included triglyceride (TG), total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL), very low-density lipoprotein (VLDL). TC, LDL, HDL, VLDL cholesterol, serum triglycerides (TG) were estimated by Cholesterol Oxidase-Peroxidase method using standard kit obtained from Span Diagnostics.

Lipid peroxidation

On 14th day, all animals were killed. Their liver, heart, and kidneys were immediately excised and after washing with 0.9% NaCl, wet tissue (0.5 g) was weighed exactly and homogenized in 4.5 ml of 0.25 M sucrose using a Teflon homogenizer. The cytosolic fraction was obtained by a two-step centrifugation first at 1000×g for 10 min and then at 2000×g for 30 min at 4^{o}C.

A volume of the homogenate (0.2 ml) was transferred to a vial and was mixed with 0.2 ml of a 8.1% (w/v) sodium dodecyl sulfate solution, 1.5 ml of a 20% acetic acid solution (pH 3.5), and 1.5 ml of a 0.8% (w/v) solution of thiobarbituric acid and the final volume was adjusted to 4.0 ml with distilled water. Each vial was tightly capped and heated in boiling water bath for 60 min. After cooling, equal volumes of tissue blank or test sample and 10% trichloroacetic acid were transferred into a centrifuge tube and centrifuged at 1000×g for 10 min. The absorbance of the supernatant fraction was measured at 532 nm [18].

Statistical analysis

All values are expressed as mean ± S.E.M. (n= 6) the results were considered statistically significant if *P<0.01*. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student T-test. Each value is mean ± SEM (n = 6)*, *P<0.01*, when compared to corresponding value of normal control, ** *P<0.01*, when compared to corresponding value of diabetic control.

RESULTS AND DISCUSSION

Results of the effect of ACME, Cadambine, and glibenclamide on fasting blood glucose level (FBG), triglyceride (TG), total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL), very low-density lipoprotein (VLDL), change in body weight, and lipid peroxide level (TBRAS) are presented in **tables 1-8**.

This study was undertaken to evaluate the hypoglycemic and antioxidant potential of ACME, Cadambine, in alloxan-induced diabetic rats. ACME, Cadambine, showed remarkable better activity. Alloxan produced significant loss in body weight as compared to the normal animals during the study. Diabetic control continued to lose weight till the end of the study, while ACME at two doses (200 and 400 mg/kg) showed significant improvement in body weight as compared to diabetic control. Cadambine showed a significant (P<0.01) reduction in triglycerides, LDL, and VLDL. High density lipoprotein content increased significantly (P<0.01) in Cadambine treated diabetic animals even more then treated with glibenclamide. There was significant elevation in the level of TBARS in diabetic control as compared with the corresponding normal control. However, the oral administration of ACME and glibenclamide tended to bring these values back to normal (Tables 1-8).

In acute toxicity study, ACME treated animals did not show any change in their behavioral pattern. There was no significant difference in the body weights and food consumption when compared to the vehicle treated group. Alloxan significantly induced hyperglycemia; Oral administration of test samples for 14 days caused a significant decrease in blood glucose levels. The possible mechanism by which ACME mediated its antidiabetic effect could be by potentiation of pancreatic secretion of insulin from existing b-cells of islets in the extract treated animals. The hypoglycemic activity of ACME was compared with glibenclamide, a standard hypoglycemic drug.

Table 1: Effect of treatment on fasting blood glucose level (FBG)

Experimental Groups	Mean value of blood glucose concentration (mg/dl)	
	Day1	Day 14
Normal Control	85.1± 2.31	86.5± 1.18
Diabetic Control	241.2 ± 0.81*	248.6 ± 1.10*
Diabetic + ACME (200 mg/kg)	239.0 ± 1.71**	124.5 ± 1.74**
Diabetic + ACME (400 mg/kg)	238.8 ± 0.89**	117.7 ± 0.95**
Diabetic + Cadambine (10mg/kg)	246.1 ± 2.11**	97.3 ± 1.01**
Diabetic + Glibenclamide (10 mg/kg)	242.0 ± 2.51**	115.1 ± 2.13**

Table 2: Effect of treatment on triglyceride level (TG)

Experimental Groups	Triglyceride (mg/dl)	
	Day1	Day 14
Normal Control	67.5 ± 1.23	86.0 ± 2.31
Diabetic Control	149.6 ± 1.33*	210.8 ± 1.25*
Diabetic + ACME (200 mg/kg)	154.8 ± 1.39**	128.7 ± 0.95**
Diabetic + ACME (400 mg/kg)	168.0 ± 0.85**	115.6 ± 1.19**
Diabetic + Cadambine (10mg/kg)	158.7± 1.29**	103.5 ± 1.33**
Diabetic + Glibenclamide (10 mg/kg)	156.68 ± 1.52**	104.5 b ± 1.87**

Table 3: Effect of treatment on total cholesterol level (TC)

Experimental Groups	Total cholesterol (mg/dl)	
	Day 1	Day 14
Normal Control	56.1± 0.76	86.9± 0.901
Diabetic Control	102.9± 0.51*	119.2 ± 0.44*
Diabetic + ACME (200 mg/kg)	98.5 ± 0.72**	98.1 ± 1.13**
Diabetic + ACME (400 mg/kg)	87.7± 2.71**	65.5 ± 3.19**
Diabetic + Cadambine(10mg/kg)	111.5 ± 1.57**	57.2 ± 2.11**
Diabetic + Glibenclamide (10 mg/kg)	99.5 ± 1.43**	57. 6 ± 2.86**

Table 4: Effect of treatment on high density lipoprotein level (HDL)

Experimental Groups	High density lipoprotein (mg/dl)	
	Day 1	Day 14
Normal Control	43.7± 0.49	46.0± 1.59
Diabetic Control	40.1± 2.11*	36.4 ± 1.05*
Diabetic + ACME (200 mg/kg)	42.0 ± 1.62**	44.4 ± 0.42**
Diabetic + ACME (400 mg/kg)	38.7 ± 1.69**	46.7 ± 1.53**
Diabetic + Cadambine (10mg/kg)	43.2 ± 1.01**	51.5 ± 2.01**
Diabetic + Glibenclamide (10 mg/kg)	38.8 ± 1.34**	46.6 ± 1.21**

Table 5: Effect of treatment on low density lipoprotein level (LDL)

Experimental Groups	Low density lipoprotein (mg/dl)	
	Day 1	Day 14
Normal Control	26.9 ± 1.36	28.8± 1.49
Diabetic Control	53.7 ± 2.32*	65.8± 2.01*
Diabetic + ACME (200 mg/kg)	52.9 ± 3.10**	48.4 ± 2.95**
Diabetic + ACME (400 mg/kg)	53.8 ± 1.42**	42.3 ± 2.35**
Diabetic + Cadambine (10mg/kg)	52.3 ± 2.21**	43.5 ± 2.67**
Diabetic + Glibenclamide (10 mg/kg)	55.7 ± 1.65**	39.4 ± 2.06**

Table 6: Effect of treatment on very low-density lipoprotein level (VLDL)

Experimental Groups	Very low-density lipoprotein (mg/dl)	
	Day 1	Day 14
Normal Control	12.6 ± 1.34	12.9 ± 1.25
Diabetic Control	15.9 ± 1.08*	29.9 ± 0.83*
Diabetic+ ACME (200 mg/kg)	144.9 ± 0.98**	18.9 ± 1.12**
Diabetic+ ACME (400 mg/kg)	15.7 ± 1.28**	18.3 ± 0.78**
Diabetic + Cadambine (10mg/kg)	18.9 ± 1.15**	15.8 ± 1.16**
Diabetic+ Glibenclamide (10 mg/kg)	15.1 ± 2.17**	21.1 ± 0.59**

Table 7: Effect of treatment on body weight (gm)

Experimental Groups	Change in body weight (g)	
	Day 1	Day 14
Normal Control	258.3 ± 3.52	256.5 ± 2.35
Diabetic Control	205.4 ± 2.68*	190.6 ± 1.78*
Diabetic+ ACME (200 mg/kg)	209.2 ± 1.47**	248.5 ± 2.11**
Diabetic+ ACME (400 mg/kg)	210.5 ± 2.38**	243.6 ± 2.56**
Diabetic + Cadambine(10mg/kg)	206.8 ± 2.76**	249.5 ± 1.50**
Diabetic+ Glibenclamide (10 mg/kg)	207.6 ± 1.99**	250.5 ± 3.01**

Table 8: Effect of treatment on thiobarbituric acid reactive substance (TBRAS) (µM/g)* of wet tissue

Experimental Groups	TBRAS (µM/g) of wet tissue
Normal Control	0.38 ± 0.08
Diabetic Control	0.73 ± 0.11*
Diabetic+ ACME (200 mg/kg)	0.58 ± 0.21
Diabetic+ ACME (400 mg/kg)	0.52 ± 0.10**
Diabetic + Cadambine(10mg/kg)	0.51 ± 0.43**
Diabetic+ Glibenclamide (10 mg/kg)	0.46 ± 0.08**

From the results of the present study, it may be suggested that the mechanism of action of ACME may be like glibenclamide action. Hypercholesteremia and hypertriglyceridemia are primary factors involved in the development of atherosclerosis and coronary heart disease which are the secondary complications of diabetes. ACME significantly reduced serum triglycerides and total cholesterol in alloxan-diabetic rats. Thus, it is reasonable to conclude that ACME could modulate blood lipid abnormalities.

In diabetes, tissue damage is mediated by free radicals by attacking membranes through peroxidation of unsaturated fatty acids. Lipid peroxidation eventually leads to extensive membrane damage and dysfunction. Decreased lipid peroxidation and improved antioxidant status may be one of the mechanisms by which drug treatment could contribute to the prevention of diabetic complications. In our study, ACME significantly attenuated the increased lipid peroxidation which could be due to the antioxidant effect of Cadambine.

From this study, we can conclude that aqueous extract of *Anthocephalus cadamba* and its bioactive constituent, cadambine has beneficial effects on blood glucose level. It has the potential to impart therapeutic effect in diabetes. Furthermore, studies can be carried out to explore this lead compound with the help of molecular modeling methods to understand exact mechanism.

ACKNOWLEDGEMENT

The authors wish to extend their gratitude to Prof. (Dr.) Rajendra B. Lal, Hon'ble Vice Chancellor, Sam Higginbottom University of Agriculture, Technology and Sciences, Allahabad and Prof. P. Malairajan, Head, Department of Pharmaceutical Sciences, SHUATS, Allahabad, for providing excellent research environment.

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How to cite this article:

Singh HP, Irchhaiya R, Verma A, Pandey H and Singh PP. Phytochemical analysis, exploration of antidiabetic and antioxidant potential of *Anthocephalus cadamba* (Roxb.). *Int. J. Res. Dev. Pharm. L. Sci*. 2017; 6(6): 2800-2805. Doi: 10.13040/IJRDPL.2278-0238.6(6). 2800-2805.