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RESEARCH ARTICLE

Antihyperglycemic activity of *Vernonia cinerea* L. on alloxan-induced diabetic mice.

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Abstract

The present study was carried out to isolate and identify the potent antidiabetic compounds from whole plant of *Vernonia cinerea* (VC). (Sesquiterpene lactones). was isolated from ethanolic extract of *Vernonia cinerea*. The Compound-I was undertaken to evaluate the antidiabetic activity against normal and alloxan-induced diabetic mice. Compound-I showed better reduction of antihyperglycemic activity. The standard drug glibenclamide(10mg/kg) also produced significant($p<0.05$) reduction in blood glucose level against alloxan-induced diabetic mice.

Introduction

Diabetes mellitus is the name given to a group of disorders characterized by absent or deficient insulin secretion or peripheral insulin resistance resulting in hyperglycemia. Currently the presence of abnormally high glucose levels in the blood is the only criterion on which diagnosis of diabetes mellitus is based. There is increasing demand to use the natural products with anti-hyperglycemic activity. *Vernonia cinerea* L.(Asteraceae) is an annual herbaceous plant, distributed throughout India and grown as a weed plant(Abirami *et al*.,2012). It is commonly known as 'Sahadevi' in Sanskrit and Hindi, 'Little ironweed' in English, 'Kukshim' in Bengali, 'Puvamkurunnel' in Malyalam (Patnayak *et al.*,2008).

It is an important and versatile medicinal plant playing effective role to treat cancer, abortion and various gastrointestinal disorders (Yusuf *et al.*,1994).The juice of the plant is given to children with urinary incontinence. Chloroform extract of stem-bark and leaves of *Vernonia cinerea* showed diuresis activity but methanolic extract showed antidiuretic property(Adeboye *et al*.,1997)

The present study was designed to investigate the phytochemical bioactive compounds of the ethanolic extract of *V.cinerea* and its anti-hyperglycemic activity.

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Material and Methods

Collection and Identification of plant material

V.cinerea was collected in October-November 2008 from the local surroundings of Betul and Bhopal District of M.P., India. The plant was identified and herbarium voucher specimen (No.AST/612) was deposited at Bhoj Mahavidyalaya Bhopal(M.P.)India.

Preparation of the plant extract

The whole plant of *V.cinerea* was first washed well with tap water and kept for drying in shade at room temperature and thoroughly air dried plant material was grinded to powder (40-60 mesh) weighted and stored. The powder was extracted with ethanol by soxhlet extraction method. The percentage yields of crude extract were 27.48% in ethanol.

Preliminary phytochemical screening

The qualitative phytochemical screening of ethanolic extract of *V.cinerea* plant contains glycosides, esters, flavonoids, steroids, tannins and terpanoids (Sesquiterpene lactones).Which are presented in Table-1.

Isolation and Identification of the active compound

Six gram of the pure VC plant ethanol extract was admixed with 10g silica gel (60-120 mesh),dried for

uniform mixing and the admixture was loaded in a column (5 cm diameter x 50 cm height)which was already packed with silica gel (150g) using ethyl acetate as the solvent. The supply of the solvent and combination of the solvent was replenished from a separating funnel. The various fractions thus obtained were collected in small glass vials using Benzene:Chloroform (1:1) and n-Haxane:Chloroform (3:1) solvent system. The active fraction (Fr-I) eluted at Benzene:Chloroform. The compound was obtained as brownish semisolid. The fraction was characterized by HPLC and spectroscopy techniques like ^{1}H NMR, IR, UV and MS. Melting points were determined using a Mitamura melting point apparatus and were uncorrected.

Experimental animals

Swiss albino mice (24-35g) were allowed free access to standard pellet diet and water. The experimental protocol was approved by the IAEC (Institutional Animal Ethical Committee) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animal) (Reg.No.1283/C/09/CPCSEA).

Acute toxicity studies

Acute toxicity study was carried out on plant extracts using female and male Swiss albino mice. The mice were fasted overnight and the weight of each mouse was recorded just before use. Animals were divided randomly into a control and three treatment groups, each group consisting of four mice (2 male and 2 female).Control group received only the vehicle and each treatment group received orally the 70% ethanol and aqueous extract of the studied plant in a dose of 1000, 2000 and 5000mg/kg. and then they were observed daily for three days for any change in general behavior and physical activities(Burger *et. al.*,2005, Latha *et al.*,2010).

Body weight

The change in the body weight of control and experimental groups of mice treated with ethanolic extract and compound-I of *V.cinerea* in Table 2.

Oral glucose tolerance test

After 2 weeks of treatment with the ethanol extract and compound-I, the animals were made to fast for 12- 14 hours. Their body glucose level were measured and glucose solution (2g/kg body weight) was administered orally and in a volume of 1 ml. Blood samples were collected 30, 60 and 120 minutes after administration of glucose in order to evaluate their blood glucose level(Kumar *et al.*,2006).

Antidiabetic evaluation

Induction of diabetic mellitus :After fasting for 12-14 h,40 rats were injected by intraperitoneally with a single dose of 200mg/kg alloxan monohydrate (Sigma St Louis M.O.,USA) after dissolving it in freshly prepared ice-cold citrate buffer (pH 4.5). After the injection, they had free access to feed and water and were given 5% glucose solution to drink overnight to counter the hypoglycemic shock. The development of diabetes was confirmed after 48 h of the Alloxan monohydrate injection. The rates having fasting blood glucose level more than 200mg/dL were selected for experimentation(Kumar *et al.*,2006). From the out of 40 animals, 4 animals were died before grouping and 6 animals were omitted from the study, because mild hyperglycemia (below 150mg/dL). From the 30 diabetic mice, they were divided into six groups each having 5 animals (Nagappa *et al*.,2003).

Collection of blood samples and glucose determination

Blood samples were collected by end tail vein cutting method and blood glucose level was determined by using one touch electronic glucometer using glucose strips (Lifescan,Johenson and Johenson Ltd)(Kumar *et al*.,2006).

Experimental protocol

The Group I-consist of 5 normal control animals. The remaining each group consists of 5 alloxan-induced diabetic rates.

Group I-consisted of normal rats that neither received alloxan monohydrate nor any drug,

Group II-served as positive control(diabetic control),

Group III-rates were diabetic and treated with Glibenclamide (10mg/kg p.o.),

Group IV&V-diabetic rates received compound-I at the dose of 300 and 500 mg/kg p.o. respectively,

Group VI & VII- diabetic rats received (crude-I) ethanolic extract at the dose of 300 and 500 mg/kg p.o. respectively.

All the group of animals received the treatment by the above schedule for 14 days. Blood samples were collected one hour after drug administration on before treatment and day 01, 07 and 14th day to determine the blood glucose level by electronic glucometer (Babu *et al*.,2002).

Statistical analysis

Data were statistically evaluated by use of one way ANOVA, followed by post hoc Schiff's test using version 13 of SPSS software and Microsoft Office Excel 2003. The values were considered to be significant if $p<0.05$ was obtained.

Table-1. Qualitative phytochemical screening of ethanolic extract of *V. cinerea* L.

S. NO.	Chemical constituents	Tests	Results
01.	Test for Alkaloids	Mayer's Test	-
		Wagner's Test	-
		Dragendroff's Test	-
		Hager's Test	-
02.	Test for Amino acids	Million's Test	-
		Ninhydrin Test	-
03.	Test for Corbohydrates	Benedict's Test	+
		Fehling's Test	-
		Molisch's Test	-
04.	Test for Glycosides	Keller killani Test	+
05.	Test for Gums	Molisch's Test	-
06.	Test for esters	Zeisel Test	+
07.	Test for Flavonoids	Shinoda Test	+
08.	Test for Saponins	Foam Test	-
09.	Test for Steroids	Liebermann-Burchard Test	+
		Sulphuric acid Test	+
10.	Test for Tannins	Ferric chloride Test	+
11.	Test for Terpanoids	Liebermann-Burchard Test	-
		Salkowski Test	+

Key: + : Positive, - :Negative

Table-2. Showing change in body weight of mice treated with Compound-I and Ethanolic extract of *V.Cinerea*

Groups	Doses (mg/kg)	Days of treatment			% Change 1	% Change 2
		Day 0	Day 1	Day 14		
Crude-I (Ethanol)	300	30.86±1.21	28.76±1.16	29.52±1.39	-6.8	2.64
Crude-I (Ethanol)	500	30.66±1.20	28.52±1.26	29.35±1.23	-6.98	2.91
Compound-I	300	26.68±0.58	25.00±0.96	25.76±0.85	-6.29	3.04
Compound-I	500	31.94±1.00	29.42±1.24	30.38±1.43	-7.89	3.26
Normal Control (Negative)	1 ml (vehicle)	25.00±0.96	25.38±0.99	25.52±0.96	1.52	0.55
Diabetic Control (Positive)		31.92±1.35	29.66±1.09	28.62±1.33	-6.45	-4.15
Glibenclamide	10	30.74±1.18	28.96±1.27	30.20±0.65	-5.79	4.28

Each result is with a mean of 5 mice. %change1 indicates the change between day 0 (before alloxan-induction) and day 1 (after alloxan-induction).% change2 indicates the change between day 1 and day 14.

Results

Identification of the compound

After isolation of the ethanol extract, a brownish semisolid was obtained. Structural determination of the Compound-I was done using spectroscopy technique and it was confirmed as a sesquiterpene lactone. The % yields of sesquiterpene lactone was 0.82% in *V.cinerea* whole plant powder. The Compound-I was identified based on the following evidence:

HPLC- HPLC analysis of column purified fraction – I Fifteen peaks were found at 13.45 min.

IR spectra - 3752 cm^{-1}, 3446 cm^{-1}, 2927 cm^{-1}, 2337 cm^{-1} ,1643 cm^{-1}, 1223 cm^{-1}, 770 cm^{-1}

^{1}H NMR spectra- δ 0.89 (3H, s, H-20), δ 0.89 (3H, s, H-20), δ 0.99 (3H, s, Me), δ 1.07 (3H, s, H-19), δ 1.25 (3H, s, H-18), δ 1.96 (3H, s, H-16), δ 2.77 (2H, m, H-2), δ 4.32 (2H, d, J = 9.0 Hz, H-15), δ 5.34 (1H, s, H-17), δ 5.36 (1H, s, H-17), δ 7.26 (1H, t, J=9 Hz, H-14).

Mass spectra -Concentrated mass major peak were found of molecular weight 308 m/z.

UV spectra -Active Fr. – I maxima found at 254 nm, 280 nm, 470 nm, 510 nm.

On the basis of spectral analysis, the molecular formula of Compound-I may be $C_{20}H_{30}O_3$ by mass analysis (m/z 308).

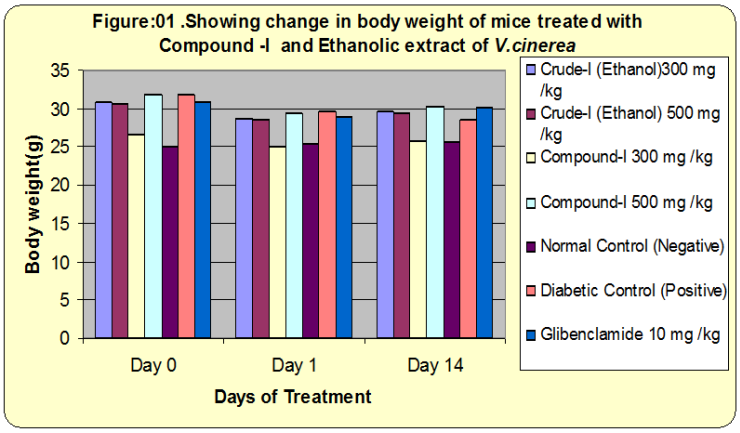
Melting point of compound (I): 117 ^{0}C to 118 ^{0}C

Acute toxicity studies

Acute toxicity studies conducted revealed that the administration of graded doses of both the crude petroleum either and 70% ethanol extracts(up to a dose of 5000mg/kg body weight)of *V.cinerea* did not show any significant toxicity. Plant extracts up to 5000mg/kg did not produce significant changes in behaviors such as alertness, motor activity, breathing, restlessness, diarrhea, convulsions, coma and appearance of the animals. No death was observed up to the 5g/kg body weight for the crude extracts of plant. These effects were observed during the experimental period (72 hrs).The results showed that in single dose, the plant extracts had no adverse effect, indicating that the medium lethal dose(LD50),could be greater than 5g/kg body weight in mice.

Body weight

The change in the body weight of control and experimental groups of mice treated with ethanolic extract and compound-I isolated from *V.cinerea* is shown in Table- 2. Alloxan-induced (200mg/kg body weight) mice showed loss in body weight (From 6.29 to 7.89%), which was reversed by oral administration of compound-I and ethanol extract of *V.cinerea*. The body weight of the normal control mice (Negative control), which took the vehicle only, did not show any significant difference, i.e. a 0.55% change on the 14th day. However, the body weight of diabetic control mice (Positive control) showed a 4.15% decrease in their body weight after two weeks. In the untreated diabetic control group out of the five animals one mouse died on the tenth day. A dose-dependent body weight improvement was observed starting from day 1 in diabetic mice treated with both compounds-I and ethanol extracts . The effect was more pronounced in case of the compound-I treated mice (3.04-3.26%) as compared with the respective dose of the crude ethanol extract (2.64-2.91%)effect during the experimental period. During a 14-day treatment, the compound-I at a dose of 500 mg/kg showed a significant increase in the body weight of the mice from 29.42 ± 1.24 g on day 1 to 30.38 ± 1.43 g (3.26% increment) on day 14.



Antidiabetic activity

The blood sugar levels measured in normal and experimental rats in initial and at the 01, 07 and 14 th days of treatment are given in table-3.Alloxan induced diabetic rates show significant increase in the levels on blood glucose as compared to normal rates.Oral administration of Compound-I and crude ethanol extract (300 and 500 mg/kg)showed significant decrease ($p<0.05$) in blood glucose level. The isolated Compound-I, Sesquiterpene lactone may be Hirsutinolide type (Kuo *et al.*,2003) at a dose level of 500mg/kg showed better reduction ($p<0.05$) in blood glucose level compared to similar dose of crude ethanol extract. The standard drug glibenclamide decreased blood glucose level in 14 days treatment. On treatment with compound -I (300 and 500 mg/kg), the fasting mean blood glucose levels on day-1 (after being diabetic), i.e. 335.6 ± 14.01 mg/dl reduced to 221.00±16.20mg/dl and

326.60 ± 22.23 mg/dl reduced to 174.20 ± 17.92 mg/dl respectively. This reduction accounts for 34.14% and 46.66%, respectively. The fasting mean blood glucose level of diabetic mice treated with glibenclamide showed a reduction of 51.05% as compared with diabetic control (Positive control) mice on day 14.The improvement in blood glucose homeostasis was in dose dependent manner after 14 days treatment. The effect of the compound-I at a dose of 500 mg/kg body weight showed significantly better reduction as compared with the respective compound-I at a dose of 300 mg/kg and also with that of ethanol extract. This study indicated that the reduction of blood glucose level in compound-I and crude ethanol extract of *V.cinerea* in alloxan-induced mice were a dose dependent.

Table-3. Showing effect of Compound-I (purified from *Vernonia cinerea* L.)and Ethanolic crude extract on fasting blood glucose level (mg/dl) in normal control and alloxan-induced diabetic mice

Groups	Days of treatment			
	Day 0	Day 1	Day 7	Day 14
Normal control (Negative control)	110.00±11.47	105 .40 ± 10.99	111.40 ± 10.94	109.80 ±7.49
Diabetic control (Positive control)	123.40± 9.29	371.20 ± 37.20*	391.80 ± 31.26*	405.00 ±40.97*
Glibenclamide 10mg/kg	116.40 ± 3.97	349.40 ± 27.57	285.00 ± 22.49*	171.00± 18.29*
Crude-I 300mg/kg	127.80±5.31	370.00±19.46	313.40±14.39*	270.20±18.94*
Crude-I 500mg/kg	125.60±4.56	249.40±21.56*	213.20± 20.36*	173.00±13.19*
Compound-I 300mg/kg	119.20 ± 4.66	335.60 ± 14.01	290.40 ± 26.56*	221.00± 16.20*
Compound-I 500mg/kg	115.00 ± 4.42	326.60 ± 22.23*	241.20 ± 10.16*	174.20± 17.92*

Values are given as mean ± standard deviation for groups of five animals. Values are statistically significant at* $p<0.05$.

Table-4. Showing glucose tolerance test of compound-I and ethanolic extract of *V.cinerea* on alloxan-induced diabetic mice

Groups	Time intervals			
	Base line	30 min.	60 min.	120 min.
Compound-I (300mg/kg)	221±16.2	284.2±24.93	312.6±26.12	256.8±18.42
Compound-I (500 mg/kg)	174.2±17.92	251.6±20.98	266.2±13.83	181.8±3.02
Crude-I ethanol extract (300 mg/kg)	270.20±18.94	301.4±14.72	321.2±17.68	282±23.86
Crude-I ethanol extract (500 mg/kg)	173.00±13.19	254.2±12.93	273.4±13.81	218.60±3.02
Normal Control	109.8±7.49	151.6±14.96	156.8±7.67	131.4±10.22
Diabetic Control	405±40.97	422±26.65	433.8±30.12	432.6±29.54
Glibenclamide(10 mg/kg)	171±18.29	201.4±18.92	214.8±21.21	173.8±15.31

Values are given as mean ± standard deviation for groups of five animals.

Oral glucose tolerance test

On fasting, the blood glucose level of the mice demonstrated basal hyperglycemia (Figure-03). The mean blood glucose value in the normal control (Negative control) mice rose to a peak value 60 min. after glucose load and decreased to near normal level at 120min .In diabetic control(Positive control) mice, however, the peak increase in mean blood glucose concentration was observed after 60 min and remained high over the next 60 min. The animals that were subjected to oral glucose tolerance test showed a reduction in the mean blood glucose levels after 60 min. load of glucose. At 60 min. the blood glucose level reached the maximum in Compound-I treated animals and than significant reduction was observed in the blood glucose level of diabetic treated with glucose loaded mice as compared with diabetic control (Positive control) mice, loaded only glucose. The mean blood glucose level at 120 min. after glucose administration was near to the baseline (fasting) in the Compounds-I treated as compared with diabetic mice untreated (Positive control).(Table-4)

Figure-02.Showing effect of Compound-I (purified from *Vernonia cinerea* L.)and Ethanolic crude extract on fasting blood glucose level (mg/dl) in normal control and alloxan-induced diabetic mice

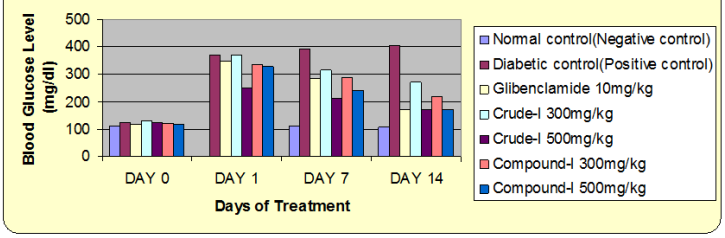
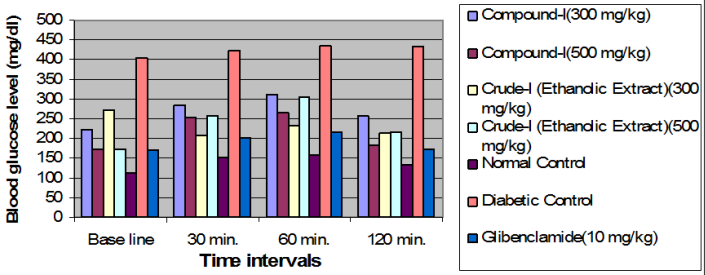


Figure-03:Showing glucose tolerance test of compound-I and ethanolic extract of *V.cinerea* on alloxan-induced diabetic mice



Discussion

The aim of the present study was to evaluate the antidiabetic effect of ethanolic extract of *V.cinerea* plant and isolated compound-I(Sesquiterpene lactone), may be Hirsutinolide type(Kuo *et al.*,2003) against alloxan induced diabetic rates. The continuous treatment of the extracts of *V.cinerea* for a period of 14 days produced a significant reduction in the blood glucose level of mice. These results confirmed the use of *V.cinerea* plant in traditional practice as an antidiabetic agents and for treatment of many diseases. The standard drug, Glibenclamide has been used for many years to treat diabetes, to stimulate insulin secretion from pancreatic β cells(Kumar *et al.* ,2008). It may be suggested that the mechanism of action of sesquiterpene lactone (Compound-I) is similar to glibenclamide, this is may be the first report that demonstrate antidiabetic properties for this compound in *V.cinerea* plant.

The possible mechanism by which plant extract brings about a decrease in blood sugar level may be by potentiation of the insulin effect of plasma by increasing either the pancreatic secretion of insulin from β cells of the islets of Langerhans or its release from the bound form, A number of other plants have been reported to exert hypoglycemic activity through insulin release stimulatory effects(Gupta *et al.*,1994, Kumar *et al.*,2008).

In the tribe *Vernonieae* (Family-Asteraceae) sesquiterpene lactones(Lopes, 1991) are found, for which the different biological effects are described. The data suggest that these compounds have anti-inflammatory and antiulcer (Feltenstien *et al.*,2004), antimalarial(Chung and Moon,2009) and antibacterial properties(Ntutelaa *et al.*,2009, Saroglou *et al.*,2010). These result could be related to the α, β -unsaturated corbonyl groups in these compounds. Which seems to be important for their cytotoxicity(Lee *et al.*,1971, Kreuger *et al.*,2012).

A wide range of chemical compounds including steroids, flavonoids, sesquiterpene lactones(Chopra *et al.*,1992), esters, triterpenoids(Harborne and Baxer,1996) and glycosides have been isolated from *V.cinerea*. Extracts and metabolites from this plant have been known to possess pharmacological properties (Buskuhl *et al.*,2010).

The ethanolic extracts *V.cinerea* evaluated in this work was phytochemically investigated in parallel,

revealing the presence of carbohydrates, (terpanoids) sesquiterpene lactones (may be Glaucolides or Vernolide A&B and related hirsutinolides) , glycosides, flavonoids, esters, steroids, tannins.

These results confirmed the use of *V.cinerea* in traditional system of medicine to treat diabetes in India. Further comprehensive chemical and pharmacological investigations are needed to elucidate the exact mechanism of the anti-hyperglycemic effect of *V.cinerea* plant.

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