Asian Journal of Pharmaceutical and Clinical Research
Vol. 4, Issue 2, 2011 ISSN - 0974-2441

Research Article

PHYTOCHEMICAL INVESTIGATION AND ANTI-DIABETIC ACTIVITY OF *ADHATODA ZEYLANICA*

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ABSTRACT

Since ancient times, plants have been an exemplary source of medicine. Ayurveda and other Indian literature mention the use of plants in treatment of various human ailments. India has about 45 000 plant species and among them, several thousands have been claimed to possess medicinal properties. "A medicinal plant is any plant which, in one or more of its organ, contains substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs."*Adhatoda zeylanica* is indigenous to India, where it is found in sub-Himalayan track upto an altitude of 1000 m and in Maharashtra especially, in Kankan region. Besides India, it is found in Myanmar, Srilanka and Malaya. The leaves, flowers, fruits and roots are extensively used for treating cold, cough, whooping cough, chronic bronchitis and asthma. It has also aroused considerable interest for its beneficial effects in malaria, dysentery, diarrhea, antimicrobial, anthelmintic. and antiperiodic. Owing to their immense importance and varied bioactivities exhibited by *Adhatoda zeylanica*, efforts have been made from time to time to generate libraries of isolated compounds and screen them for potential biological activities.Chemical constituents of *Adhatoda zeylanica* (MP-1 to MP-4) were isolated successfully with higher yield. The structures of isolated compounds were confirmed by the use of spectral data UV, FTIR, ^{1}H NMR, Mass and HPLC. The extract of *Adhatoda zeylanica* was evaluated for antidiabetic activity.

Key words: *Adhatoda zeylanica,* Hypoglycemic agents, Antidiabetic.

INTRODUCTION

Diabetes mellitus is a major disease characterized by derangement in carbohydrate, fat and protein metabolism, affecting nearly 10% of the population. In the recent past many hypoglycemic agents are introduced, still the diabetes and the related complications continue to be a major medical problem not only in developed countries but also in developing countries.

Many Indian medicinal plants are reported to be useful in diabetes, however, search for new anti-diabetic drugs continue.[1, 2]. Several drugs such as biguanides and sulfonylureas are presently available to reduce hyperglycaemia in diabetes mellitus. These drugs have side effects and thus searching for a new class of compounds is essential to overcome these problems[3]. Management of diabetes without any side effects is still a challenge to the medical community. There is continuous search for alternative drugs. Therefore, it is prudent to look for options in herbal medicine for diabetes as well.

Traditional antidiabetic plants might provide new oral hypoglycaemic 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. Medicinal plants like *Trigonella foenum graecum*, *Allium sativum*, *Gymnema slyvestre* and *Syzigium cumini* have been studied 4 for treatment of diabetes mellitus. However, detailed studies on the efficacy, mechanism of action and safety of plant extracts are needed.[4]So it has been thought worthwhile to perform phytochemical and pharmacological studies on *Adhatoda zeylanica*.

From the root of the plant, isolation and characterization of vasicol has been reported.[5] 2', 4 – Dihydroxychalcone 4- glucoside has been identified in the flowers of *Adhatoda zeylanica*[6]

MATERIAL AND METHOD

The experimental work was carried out on leaves of Adhatoda *zeylanica*

Collection & Authentication of plant material

The leaves and twigs of *Adhatoda zeylanica* were collected from Srinagar Garhwal, district Pauri, Uttrakhand, India in the month of December. The plant was identified by Botenical survey of India, Northern regional centre, Dehradun (BSD) with the accession number BSD-112751.

Successive Solvent Extraction

Air-dried and powdered leaves of *Adhatoda zeylanica* 500g were extracted successively with different solvents like hexane, petroleum ether (40-60°), benzene, acetone, chloroform, ethanol and methanol in a soxlet apparatus for 48 hrs. Each time before extracting with the next solvent, the powdered material was dried in the hot air oven below 50°C. The extracts were concentrated by distillating off the solvent.

Column chromatography of compounds

Silica gel (60-120 mesh) was used as absorbent for column chromatography. The column was taken and packed with cotton at the bottom of the column. The slurry was prepared by silica gel and chloroform was used as solvent for free flowing consistency. It was poured slowly from the top of the column of the apparatus in a little quantity allowing for the even and uniform packing.

The 2/3 of column was packed by using above procedure. The extract was dissolved in the minimum quantity of ethanol and chromatographed over silica gel. It was then eluted with different solvents in increasing order of polarity eg. hexane, petroleum ether, benzene, acetone, chloroform, alcohol and water. The fractions were collected and marked. The marked fractions were subjected to thin layer chromatography to check homogeneity of various fractions.

Isolation of compound MP-1 from F_4

The Brownish residue obtained from methanolic extract was dissolved in minimum amount of chloroform in round bottom flask and absorbed over silica gel mesh size 60-120 to make slurry. The slurry was dried under reduced pressure on rotavapour and finally dried under high vacuum.

The completely dried silica gel slurry containing the methanolic extract of the drug was poured in the silica column (mesh size 60-120, 30x2cm) packed in pure column of hexane and then eluted successively with different solvent, in increasing order of polarity. The fractions collected in conical flask were marked. The marked fractions were subjected to thin layer chromatography in order to check the homogeneity of various fractions (having same R_f values). The fractions having same R_f values were combined together and concentrated. Elution of column of methanolic extract with hexane: petroleum ether (40-60°) (8:2) fraction (F_4) furnished as yellowish amorphous powder of MP-1, recrystallized from ethanol and gave

positive Dragendroff's test for alkaloid.It was obtained as yellow crystal, m.p.197-198° C, TLC Solvent system, methanol: water (6:4 v/v), R_f 0.7, UV (λ_{max}) 270 nm, Yield 120 mg (0.024 %).

The air dried leaves of *Adhatoda zeylanica* (60gm) were crushed and powdered and then extracted in Soxhlet in increasing order of polarity of solvent (Hexane, Chloroform and methanol) .The obtained extracts were concentrated and solvent was removed in rotary evaporator.

The methanolic extract was subjected for separation by column chromatography over silica gel (mesh size 60-120). For the ethanolic extract the air- dried leaves (60 gm) of *A.* were crushed & powdered and extracted with ethanol (85%) in soxhlet . The solvent was removed in rotary evaporator.For the aqueous extract the leaves (60 gm) was extracted with distilled water under reflux condenser for 16 hrs and then concentrated in rotary evaporator.

Isolation of compound MP-2 from F_6

The Brownish residue obtained from methanolic extract was dissolved in minimum amount of chloroform in round bottom flask and absorb over silica gel mesh size 60-120 to make slurry. The slurry was dried under reduced pressure on rotavapour and finally dried under high vacuum.

The completely dried silica gel slurry containing the methanolic extract of the drug was poured in the silica column (mesh size 60-120, 30x2cm) packed in pure column of hexane and then eluted successively with different solvent, in increasing order of polarity.

The fractions collected in conical flask were marked. The marked fractions were subjected to thin layer chromatography in order to check the homogeneity of various fractions (having same R_f values). The fractions having same R_f values were combined together and concentrated.

Elution of column of methanolic extract with petroleum ether (40-60°) fraction (F_6) furnished as pale yellow amorphous powder of MP-2, recrystallized from ethanol and gave positive Dragendroff's test for alkaloid.It was obtained as pale yellow crystal, m.p. 201-204°c, TLC Solvent system, Toulene, methanol, dioxane, ammonia (1:1:25:0.5v/v), R_f 0.6, UV (λ_{max}) 281 nm,Yield 110 mg (0.022 %).

Isolation of compound MP-3 from F_{20}

The Dark brownish residue obtained from ethanolic extract was dissolved in minimum amount of chloroform in round bottom flask and absorb over silica gel mesh size 60-120 to make slurry. The slurry was dried under reduced pressure on rotavapour and finally dried under high vacuum.

The completely dried silica gel slurry containing the ethanolic extract of the drug was poured in the silica column (mesh size 60-120, 30x2cm) packed in pure column of hexane and then eluted successively with different solvent, in increasing order of polarity.

The fractions collected in conical flask were marked. The marked fractions were subjected to thin layer chromatography in order to check the homogeneity of various fractions (having same R_f values). The fractions having same R_f values were combined together and concentrated.

Elution of column of ethanolic extract with petroleum ether (40-60°): benzene (8:2) fraction (F_{20}) furnished colourless amorphous powder of MP-3, recrystallized from ethanol and gave positive Dragendroff's test for alkaloid.It was obtained as colourless crystal, m.p. 138-140°c, TLC Solvent system, Benzene: methanol (6:4 v/v), R_f 0.9, UV (λ_{max}) 270 nm, Yield 102 mg (0.020 %).

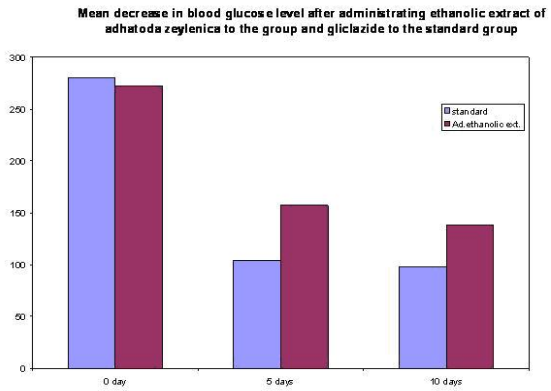
Isolation of compound MP-4 from F_{23}

The Dark brownish residue obtained from ethanolic extract was dissolved in minimum amount of chloroform in round bottom flask and absorb over silica gel mesh size 60-120 to make slurry. The slurry was dried under reduced pressure on rotavapour and finally dried under high vacuum. The completely dried silica gel slurry containing the ethanolic extract of the drug was poured in the silica column (mesh size 60-120, 30x2cm) packed in pure column of hexane and then eluted successively with different solvent, in increasing order of polarity. The fractions collected in conical flask were marked.

The marked fractions were subjected to thin layer chromatography in order to check the homogeneity of various fractions (having same R_f values). The fractions having same R_f values were combined together and concentrated. Elution of column of ethanolic extract with benzene: acetone (9:1) fraction (F_{23}) furnished as pale yellow amorphous powder of MP-4, recrystallized from ethanol and gave positive Dragendroff's test for alkaloid.It was obtained as pale yellow crystal, m.p. 278-280°c, TLC Solvent system, Methanol: water (3:2 v/v), R_f 0.8, UV (λ_{max}) 222 nm, Yield 108 mg (0.021 %).

Table 1: Mean ± SEM of blood glucose level of all the groups

Groups	After inducing alloxan	After treatment		
		0 Day	5th Day	10th Day
Normal	110±2.887	110± 2.887	112.5± 2.513	111.5± 3.138
Control	118±4.42	297± 8.914	302± 8.406	306± 7.706
Standard	305±10.58	280.5± 10.664	104± 5.586	97.5± 4.121
Test	288±8.40	272± 10.132	157± 7.257	138.5± 6.541



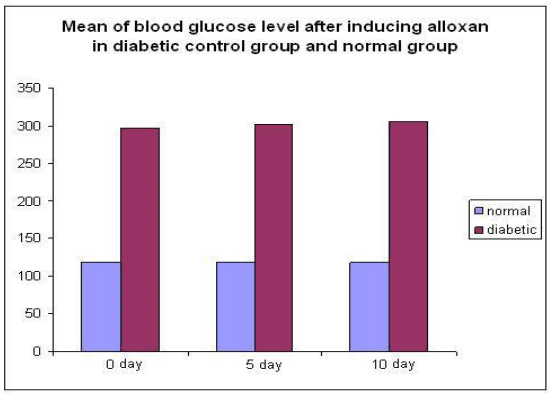


Table 2: Results Of phytochemical INVESTIGATION of various extracts

Chemical Tests	Extracts			
	Petroleum ether (40-60°)	Ethanol	Methanolic	Chloroform
1. Test of Carrbohydrates				
Fehling's test	+	+	+	+
Benedict's test	+	+	-	+
2. Test for Proteins				
Xanthoproteic test	+	-	+	-
Biuret test	+	+	-	+
Ninhydrin test	+	-	+	-
3. Test for Steroids				
Salkowski test	+	+	+	+
Liberman-Buchard test	+	+	+	+
4. Test of Glycosides				
Baljet's test	-	-	-	-
Legal's test	-	-	-	-
Kellar Killiani test	-	-	-	-
Libermann's test	-	-	-	-
5. Test for Flavonoids				
Ferric chloride test	-	-	-	-
Shinoda test	-	-	-	-
6. Test for Alkaloids				
Dragendroff test	-	+	+	-
Mayer test	+	+	+	-
Hanger's test	-	+	+	-

CHARACTERIZATION OF ISOLATED COMPOUNDS:

Characterization of compound MP-1

The compound MP-1 obtained as yellow crystals having m.p.197-198°C and gave positive test for alkaloid.TLC Methanol: water (6:4 v/v), R_f 0.7, UV λmax 270 (0.857) nm.Yield 120 mg (0.024 %), HPLC Solvent system, methanol: water (4:6 v/v) RT (min) 3.12, IR (KBr)υ: 3416.2 (O-H stretching), 3065.3 (Ar C-H stretching), 2930.2 (C-H stretching), 1613.4 (C=N stretching), 1584.1 (C=C stretching), 1299.6 (C-N stretching), 1164.7 cm^{-1} (C-O stretching).MS showed $(M)^+$ peak at m/z 188. ^{1}HNMR (DMSO): δ 2.66 (s, 2H, CH_2), 3.23 (s, 2H, CH_2), 4.23 (s, 2H, CH_2), 5.23 (s, 1H, OH), 7.42-7.83 ppm (m, 4H, Ar-H).Conclusion: On the basis of spectral evidence of the compound MP-1 was established as vasicine.

Characterization of compound MP-2

The Compound MP-2 obtained as pale yellow crystals having m.p. 201-204°C and gave positive test for alkaloid.TLC Toulene: methanol: dioxane: ammonia (1:1:25:0.5v/v), R_f 0.6, UV λmax 282 (0.047) nm, Yield110 mg (0.022 %), HPLC Solvent system, methanol: water (4:6 v/v), RT (min) 3.13, IR (KBr)υ: 3216.2 (O-H stretching), 3020.1 (Ar-C-H stretching), 2926.5 (C-H stretching), 1726.5 (C=O stretching), 1603.9 (C=C stretching), 1603.9 (C=N stretching), 1215.8 (C-N stretching), 1144.5 cm^{-1} (C-O stretching).MS showed $(M)^+$ peak at m/z 202. ^{1}HNMR (DMSO): δ 2.57 (s, 2H, CH_2), 4.45 (s, 2H, CH_2), 5.40 (s, 1H, OH), 7.13-7.40 ppm (m, 4H, Ar-H).

Conclusion: On the basis of spectral evidence of the compound MP-2 was established as vasicinone.

Characterization of compound MP-3

The compound MP-3 obtained as colourless crystals having m.p. 138-140°C and gave positive test for alkaloid.TLC Benzene: methanol (6:4 v/v), R_f 0.9, UV λmax222(0.117) nm.Yield 102 mg

(0.020 %)HPLC Solvent system, methanol: water (4:6 v/v), RT (min) 3.19, IR (KBr)υ: 3066.3 (Ar C-H), 2933.2 (C-H stretching), 1690.8 (C=O stretching), 1613.9 (C=N stretching), 1541.8 (C=C stretching), 1300 cm^{-1} (C-N stretching).MS showed $(M)^+$ peak at m/z 200, ^{1}HNMR (DMSO): δ 2.57 (s, 2H, CH_2), 4.45 (s, 2H, CH_2), 5.40 (s, 2H, CH_2), 7.13-7.40 ppm (m, 4H, Ar-H).

Conclusion: On the basis of spectral evidence of the compound MP-3 was established as deoxyvasicinone.

Characterization of compound MP-4

The compound MP-4 obtained as pale yellow crystals having m.p. 278-280°C and gave positive test for alkaloid.TLC Methanol: water (6:4 v/v), R_f 0.9, UV λmax 270 (0.832) nm, Yield 108 mg (0.021 %), HPLC Solvent system, methanol: water (4:6 v/v), RT (min) 3.12, IR (KBr)υ: 3221.0(O-H stretching), 3021.0 (Ar C-H stretching), 2926.1 (C-H stretching), 1680.8 (C=O stretching), 1590.5 (C=N stretching), 1541.3 (C-C stretching), 1216.2 cm^{-1} (C-N stretching). MS showed $(M)^+$ peak at m/z 218.^{1}HNMR (DMSO): δ 2.48 (s, 2H, CH_2), 4.31 (s, 2H, CH_2), 5.29 (s, 1H, OH), 7.21 (s, 1H, Ar-OH), 7.22-7.67 ppm (m, 3H, Ar-H),

Conclusion: On the basis of spectral evidence of the compound MP-4 was established as vasicinolone.

Experimental animals

Wistar albino rats (150–200 g) of both sexes were obtained from the experimental animal facility of S.G.R.R.I.T.S. Dehradun. Before and during the experiment, rats were fed with standard diet (Indian Feed, Chandigarh). After randomization into various groups and before initiation of experiment, the rats were acclimatized for a period of 7 days under standard environmental conditions of temperature, relative humidity, and dark/light cycle (CPCSEA

Registration No. -003/ph/09/CPCSEA). Animals described as fasting were deprived of food and water for 16 h ad libitum. The animal will be grouped as follows -Group-1(Sham control group) No treatment and no induction of Diabetes.Group-2 (Positive control) Only Diabetes will be induced in animals but no treatment will be given to them. Group-3 (Active control) Diabetic animals and standard treatment. Group-4 Diabetic animals and ethanolic extract.

Sample collection

Blood samples were collected by retro-orbital plexus puncture method

(1) Induction of diabetes

The animal will be injected with alloxan monohydrate dissolved in sodium citrate buffer at a dose of 150mg /kg b.w. intraperitoneally to induce Diabetes. Diabetes will be confirmed by testing blood sugar level using glucometer. The animals with blood sugar level more than 200 mg/dl will be selected and further maintain for four days for well establishment of diabetes.

(2) Assessment of antidiabetic effect

Active control group of animals will be treated with Gliclazide at a dose of (10mg/kg b.w.). The other groups of diabetes induced animals will be treated with ethanolic extract of 500mg /kg b. w. (orally) dose. Animals will be treated for 10 days and at regular interval of time blood glucose level will be measured using glucometer.

Statistical analysis

The observation during study will be subjected to statistical analysis. All results obtained will be needed to express as mean +/- S.E.M. The data will be analyzed using one way analysis of variance (one way ANOVA) and the group means will be compared by Dunnet test. Values will be considered statistically significant when $P< 0.001$.

RESULTS

The ethanolic extracts have shown significant ($P<0.001$) increase in glucose tolerance. The results are given in Table 1 & Figure 2. The blood glucose levels were reduced considerably by the drug administration. In alloxan-induced diabetic rats also, extract have shown considerable reduction in blood glucose levels. The results are shown in Figure 1. The reduction in glucose levels is significant ($p<0.001$) in the treated animals at 0 day, 5^{th} day and 10^{th} day after drug administration (figure 2).

CONCLUSION

Adhatoda zeylanica juice is claimed to be useful in diabetes. Results of anti-diabetic activity of *Adhatoda zeylanica* extract established the scientific basis for the utility of this plant in the treatment of diabetes. The ethanol extract have shown significant reduction in blood glucose levels in both glucose loaded and alloxan induced diabetic rats. Therefore it is obvious that the fractionation with ethanol has enriched the active principles. In glucose loaded animals, the drug has reduced the blood glucose to the normal levels. It is possible that the drug may be acting by potentiating the pancreatic secretion or increasing the glucose uptake. Ethanol extract has reduced the glucose levels in prolonged treatment study. Hypercholestrolemia, hypertriglyceridemia, hyperurea have been reported to occur in alloxan diabetic rats (Gupta et.al 1984. Resmi et al 2001) and a significant increased observed in our experiment was in accordance to these studies. Repeated administration of ethanol extract had decreased the blood glucose, urea, total cholesterol and triglycerides significantly. Histopathological examination of pancreas, liver and kidney showed the recovery of damaged tissues when section of treated groups compared with diabetic control. In conclusion, *Adhatoda zeylanica* extract showed significant anti-diabetic effect in diabetic rats after oral administration. Thus the claim made by the traditional Indian systems of medicine regarding the use of leaves juice of this plant in the treatment of diabetes stands confirms. Present efforts are directed to isolate the active constituents from ethanol extract and elucidation of mechanism of action.

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