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PHYTOCHEMICAL SCREENING, *INVITRO* ANTIOXIDANT AND *IN-VIVO* ANTIDIABETIC ACTIVITY OF METHANOLIC EXTRACT OF LEAVES OF *PIMENTA DIOICA* (LINN) MERILL

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ABSTRACT

Pimenta dioica, Family: Myrtaceae, well known for its berries called Pimento, has been used as an important spice since time immemorial, for its culinary as well as medicinal qualities. It is also known as Allspice due to its intricate aroma which is a medley of aroma from spices such as Clove, Nutmeg and Cinnamon. In India, the leaves of Pimenta are used to flavor rice which gives it a typical aroma. Various compounds have been isolated from the plant which belongs to categories like phenylpropanoids, tannins, glycosides and essential oil. Diabetes mellitus (DM) is a global health problem and the incidence of DM is increasing at alarming rate all over the world. Many Indian medicinal plants have been reported to possess potential antidiabetic activity and could play important role in the management diabetes. The aim of the present study was to evaluate qualitative and quantitative phytochemical analysis, *in vitro* antioxidant activities and *invivo* anti-diabetic potentials of methanolic extract of leaf (*Pimenta dioica*) against alloxan-induced diabetes in Wistar rat's model collected from Bhopal region of Madhya Pradesh. Qualitative analysis of various phytochemical constituents and quantitative analysis of total phenolics and flavonoids were determined by the well-known test protocol available in the literature. The *in vitro* antioxidant activity of methanolic extract of the leaves was assessed against DPPH assay method using standard protocols. Rats were given Alloxan monohydrate in sterile normal saline at a dose of 120 mg/kg to induce diabetes, and glibenclamide (5mg/kg body weight) was utilized as the usual medication. Body weight and blood glucose level were assessed in this study. Phytochemical analysis revealed the presence of flavonoids, tannin and phenolic compounds, saponins and triterpenoids and steroids. The total phenolics content of leaves of methanolic extract was (69.66mg/gm), followed by flavonoids (40mg/gm). The activities of methanolic leaves extract against DPPH assay method were concentration dependent with IC 50 values of ascorbic acid and extracts 18.99 and 59.21µg/ml respectively. Oral treatment of methanolic extract of *Pimenta dioica* using rat oral needle at 200 and 400mg/kg doses significantly decreased blood glucose levels in diabetic rats than control rats and increase body wt. Hence, the chemical constituents of the plant extract might help in preventing diabetic complications and may serve as an alternative in the present armamentarium of antidiabetic drugs. Further study to substantiate the use of the plant as antidiabetic is recommended.

KEYWORDS: *Pimenta dioica*, Myrtaceae, Diabetes mellitus, Phytochemical analysis, *In vitro* antioxidant activity, *Invivo* anti-diabetic potentials.

INTRODUCTION

Diabetes mellitus is an epidemic occurring in adults throughout the world and is the leading cause of kidney failure, heart attack, blindness and lower limb amputation. It is the fourth main cause of death in most developed countries. The prevalence of diabetes is estimated to reach 330 million by the year 2025, according to International Diabetes Federation, with the greatest potential increase being in Africa and Asia. This numerical increase will occur in developing countries. By the year 2025, over 75% of people with diabetes will reside in developing countries, as compared to 62% in 1995.[1] Currently available therapies for diabetes include insulin and various oral antidiabetic agents such as sulfonylureas, biguanides and glinides. Many of them have a number of serious adverse effects; therefore, the search for more effective and safer hypoglycemic agents is one of the important areas of investigations.[2] Many herbs and plants have been described as possessing hypoglycemic activity when taken orally.[3] According to the World Health Organization, there are more than 1200

plant species worldwide used in the treatment of diabetes mellitus and substantial number of plant showed effective hypoglycemic activity after laboratory testing.[4] Recently, some medicinal plants have been reported to be useful in diabetes worldwide and have been used empirically in antidiabetic and antihyperlipidemic remedies. More than 400 plant species having hypoglycemic activity have been available in literature, however, searching for new antidiabetic drugs from natural plants is still attractive because they contain substances which demonstrate alternative and safe effects on diabetes mellitus. Most of the plants contain glycosides, alkaloids, terpenoids, flavonoids, carotenoids etc. that are frequently implicated as having antidiabetic effect.[5] However, the study of plant for hypoglycemic, antioxidant and hypolipidemic activities may give new pharmacological approaches in the treatment of diabetes mellitus.[6] Alloxan is a toxic glucose analogue, which selectively destroys insulin-producing cells in the pancreas (that is beta cells) when administered to rodents and many other animal species. This causes an insulin-dependent diabetes mellitus (called "Alloxan Diabetes") in these animals, with characteristics similar to type 1 diabetes in humans. Alloxan is selectively toxic to insulin producing pancreatic beta cells because it preferentially accumulates in beta cells through uptake via the GLUT2 glucose transporter.[7] *Pimenta dioica* belongs to the botanical spice group of Pimenta Lindl., Myrtaceae family. The evergreen dried fruits and leaves of *Pimenta dioica* tree are used worldwide as valuable spices. They are commonly known as allspice, clove pepper, English spice, Jamaican pepper, etc. Earlier the tree was grown mainly in Central America, Jamaica, Cuba, Brazil, etc, but now grown in India too. The essential oils of *Pimenta dioica* leaves and fruits are utilized in food industry - mainly meat and canning industries as well as in perfumery compositions and cosmetic products. The therapeutic properties of the essential allspice oils are anaesthetic, analgesic, antimicrobial, antioxidant, antisceptic, carminative, muscle relaxant, rubefacient, stimulant and tonic. Pimenta oil can be helpful for the digestive system, for cramp, flatulence, indigestion and nausea. Further the essential oils can help in cases of depression, nervous exhaustion, tension, neuralgia and stress and is used as natural repellent. The essential oils of the leaves and fruits of this plant are also used in perfumes, aftershaves and commercial food flavouring. The major compound of *Pimenta dioica* oil is eugenol (70-80%). In the pimenta leaf oils 1,8- cineole, α-humulene, β-caryophyllene and cadinene-derivatives were found as important constituents in higher concentrations.[8] The aim of this study was to assess the quality (types), quantity (amount), *in vitro* antioxidant activities and antidiabetic potential of the methanolic extract of *Pimenta dioica* leaves in diabetic rats.

MATERIALS AND METHODS

Plant material

The medicinal plant *Pimenta dioica* (300 gm) was collected locally from Bhopal, M.P. After cleaning, plant parts were dried under shade at room temperature for 3 days and Then in oven at 45°C till complete dryness. Dried plant parts were stored in air tight glass containers in dry and cool place to avoid contamination and deterioration. The leaves of medicinal plant *Pimenta dioica* were authenticated by a plant taxonomist in order to confirm its identity and purity.

Chemical reagents

All the chemicals used in this investigation were given by Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem. Ltd. (Mumbai, India), Hi Media Laboratories Pvt. Ltd. (Mumbai, India), and SRL Pvt. Ltd. (Mumbai, India). Glibenclamide (Unichem, Ltd), (Alkem, Mumbai); Alloxan monohydrate; only analytical-grade substances were used in the investigation.

Extraction

Plant material fattening

Plant matter from *Pimenta dioica* was crushed up and allowed to air dry at ambient temperature. Soxhlation was used to remove the substance from the shade-dried plants using petroleum ether after it had been coarsely crushed up. The substance was extracted repeatedly until it had been adequately fatted.

Extraction by soxhlation process

Pimenta dioica powder that has been defatted was thoroughly extracted with methanol using the soxhlation process. The extract evaporated beyond their boiling points. The dried crude concentrated extract was weighed in order to calculate the extractive yield. When ready for analysis, it was then put into glass vials (6 x 2 cm) and stored in a refrigerator 4°C.[9]

Phytochemical screening

According to the protocols described, phytochemical screening was done to find any bioactive compounds.[10,11] By visually seeing a colour change or the production of precipitates following the addition of specific reagents to the solution, the tests were recognized.

Total phenol measurement

The Folin Ciocalteu reagent was employed to calculate the total phenolic substance of the extracts. Gallic acid concentration (20-100µg/ml) was produced in CH_3OH. 100µg/ml plant extract concentrations were likewise made in CH_3OH, and 0.5 ml of every sample was added to the test along with 4 ml of 7.5% sodium carbonate and 2 ml of a 10 fold diluted folin Ciocalteu reagent. After parafilming the tubes, they were keep warmed at RT for 30 minutes with periodic shaking. The absorbance at 765 nm was calculated against CH_3OH as a vacant. Gallic acid's conventional regression curve was utilized to

calculate the content of phenol overall, and the results were given in milligrammes per gramme (mg/gm) of gallic acid.[12]

Total flavonoids measurement

Rutin (20 to100µg/ml) was produced in CH_3OH at various concentrations. Test samples with a polarity of 100µg/ml or close to it were created. A sample was diluted to 0.5 ml and then added to 0.15 ml of a 5% NaNO2 solution along with 2 ml of distilled H_2O. A 10% AlCl3 solution was added after 6 minutes had passed. The combination was then given 5 minutes to stand before receiving 2 ml of a 4% NaOH solution. With distilled water, the final volume was adjusted to 5ml, and then it was left to stand for an additional 15 minutes. At 510 nm, the absorbance was calculated using H_2O as the reference. Rutin's standard regression curve was employed to calculate the whole flavonoid substance.[13]

Antioxidant activity

DPPH radical scavenging activity

For DPPH assay, the method of Gulçin *et al.,* 2006.[14] was adopted. A solution of 0.1mM DPPH (4mg/100ml) in methanol was prepared and 1 ml of this solution was mixed with 1 ml of different concentrations of the different extracts. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. Ascorbic acid was used as reference standard while methanol was used as control. Reduction of the stable DPPH radical was used as a marker of antioxidant capacity of *Pimenta dioica* extracts. The change in colour was measured at 517 nm wavelength using methanolic solution as a reference solution. This was related to the absorbance of the control without the plant extracts. The percentage inhibition of free radical DPPH was calculated from the following equation: % inhibition = [(absorbance of control - absorbance of sample)/absorbance of control] × 100%. All the tests were carried out in triplicates. Though the activity is expressed as 50% inhibitory concentration (IC50), IC50 was calculated based on the percentage of DPPH radicals scavenged. The lower the IC50 value, the higher is the antioxidant activity.

Animals

Wistar rats (200±50 gm) were residence in groups of six (n=6) under regulated humidity and temperature settings (25±2 °C, 55-65%). Rats were given regular rodent food and unlimited amounts of water. Prior to the experiments, rats spent 7 days becoming used to the lab environment. Between 8:00 and 15:00 hours, all studies were conducted in a room with no background noise. Each set of studies used a different group of rats (n=6). The Institutional Animal Ethics Committee (IAEC), established by the India's Ministry of Environment and Forests, located in New Delhi to oversee and supervise the use of experimental animals, gave its approval to the animal experiments.

Induction of diabetes in rats

After overnight fasting, diabetes was induced by intraperitoneal injection of Alloxan monohydrate in sterile normal saline at a dose of 120 mg/kg and the doses were determined according to the body weight of animals. In the present study the blood glucose levels were evaluated in all the rats prior to administration of Alloxan monohydrate. On day 3 i.e. after 72 hours, the blood glucose levels were evaluated and the rats with blood glucose level >250 mg/dl were considered as diabetic and taken up for the study.[15]

Animal grouping

The animals were randomly divided into following five groups; each group consists of six animals. Animal grouping and their treatment is as follows:

Group-I: Normal Control: - treated with normal saline.

Group-II: Negative Control:-Alloxan-induced diabetic rats in which alloxan (120 mg/kg was administrated i.e. in 0.1M sodium citrate) treated with normal saline.

Group-III: Test-1: Alloxan-induced diabetic rats treated with 200 mg/kg *Pimenta dioica*

Group-IV: Test-2:-Alloxan-induced diabetic rats treated with 400mg/kg *Pimenta dioica*

Group-V: Standard: -Alloxan-induced diabetic rats treated with Glibenclamide (5mg / kg) orally.

Diabetes was induced by a single i.p. injection of alloxan a dose of 120 mg/kg body weight. Except Group I all the other 4 groups were induced with diabetes. Glibenclamide were suspended in 0.9% NaCl in warm water as vehicle solution and administered orally for 21days. The treatment schedule was begun on 4th day after diabetic induction and it was counted as 1st day of treatment. It was continued till 21 days. Body weight and level of glucose in blood were observed on 0, 3, 7, 14 and 21 day of post treatment.

Collection of blood sample and blood glucose determination

Blood samples were drawn from tail tip of rat in the study. Fasting blood glucose estimation was done on day 0, 3, 7, 14 and 21 of the study. For the estimation of blood glucose level was used where the blood glucose level were expressed in mg/dl. This method has adequate sensitivity with the advantage that a small amount of blood (1-2 µl) can be used for blood glucose analysis. Blood sample was collected by cutting the tail tips with a sharp blade and put on the glucose test strip on the glucometer.

RESULTS AND DISCUSSIONS

The crude extracts so obtained after each of the successive Soxhlet extraction process were concentrated on water bath by evaporation the solvents completely to obtain the actual yield of extraction. The percentage yield of extraction is very important in phytochemical extraction in order to evaluate the standard extraction efficiency for a particular plant, different parts of same

plant or different solvents used. The yield of extracts obtained from the leaves of the plants using petroleum ether and methanol as solvents are depicted in the Table 1. The results of qualitative phytochemical analysis of the crude powder of leaves of *Pimenta dioica* are shown in Table 2. Methanolic extracts of sample of *Pimenta dioica* showed the presence of flavonoids, tannin and phenolic compounds, saponins and triterpenoids and steroids. Quantitative phytochemical assay was performed by calculating total phenolic content (TPC) and total flavonoid content (TFC). The TPC was calculated with respect to gallic acid (standard) and TFC was then calculated with respect to rutin taken as standard. The TPC and TFC in methanolic extract were found to be 69.66 and 40 mg/gm respectively Table 3 & Fig 1, 2. DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in methanol. This free radical remains stable at room temperature and gets decreased in the presence of an antioxidant molecule, which give rise to colorless methanol solution**.** The scavenging activity of extracts and standard on the DPPH radical expressed as IC50 value of methanol was 59.21 and ascorbic acid was 18.99 Table 4. The effects of the extract on body weight in diabetic rat are shown in Table 5. All groups prior to extract administration (0 day) showed no apparent difference in body weight compared to normal control group. Significant body weight gain was recorded for P.D 200 and 400 mg/kg at the 7th day of treatment compared to diabetic control group. All doses of the extract and standard showed a significant improvement in body weight at the 14th day when compared to diabetic control. By contrast, the body weight of the diabetic control group was significantly decreased at the 14th day compared to a normal control group. All the groups of animals were affected by diabetes, which indicates blood glucose level, was slightly change for normal control group at 7th and 14th days as compared to other groups. Day 7^{th} glucose levels were significantly decreased Glibenclamide treated group (226.0217± 0.12142) when compared with inducer group at 7th and 14^{th} days. The methanolic extract of *Pimenta dioica* treated groups 200 mg/kg and 400 mg/kg was. The observed values were 243.87 ± 0.49 and 230.73 ± 7.834when compared with inducer group at 7^{th}day. The methanolic extract of *Pimenta dioica* 200 mg/kg and 400 mg/kg have been expressed dose dependent anti diabetic action when compared to normal control and standard Glibenclamide. On day 21^{st}, methanolic extract of *Pimenta dioica* treated animals at dose level of 200 & 400 mg/kg significantly decreased and maintain the blood glucose level (201.17±7.452& 165.92±5.188) when compared to control and inducer group Table 6.

Table 1: Percentage yield of crude extracts of *Pimenta dioica* extract.

S. No	Plant name	Solvent	Theoretical weight	Yield(gm)	% yield
1	*Pimenta dioica*	Pet ether	300	2.01	0.67%
2		Methanol	257	5.32	2.07%

Table 2: Qualitative phytochemical evaluation of *Pimenta dioica* extract

S. No.	Experiment	Presence or absence of phytochemical test	
		Pet. Ether extract	Methanolic extract
1.	**Alkaloids**		
1.1	Dragendroff's test	Absent	Absent
1.2	Mayer's reagent test	Absent	Absent
1.3	Wagner's reagent test	Absent	Absent
1.3	Hager's reagent test	Absent	Absent
2.	**Glycoside**		
2.1	Borntrager test	Absent	Absent
2.2	Legal's test	Absent	Absent
2.3	Killer-Killiani test	Absent	Absent
3.	**Carbohydrates**		
3.1	Molish's test	Absent	Absent
3.2	Fehling's test	Absent	Absent
3.3	Benedict's test	Absent	Absent
3.4	Barfoed's test	Absent	Absent
4.	**Proteins and Amino Acids**		
4.1	Biuret test	Absent	Absent
5.	**Flavonoids**		
5.1	Alkaline reagent test	Absent	Present
5.2	Lead Acetate test	Absent	Present
6.	**Tannin and Phenolic Compounds**		
6.1	Ferric Chloride test	Absent	Present
7.	**Saponin**		

7.1	Foam test	Present	Absent
8.	**Test for Triterpenoids and Steroids**		
8.1	Salkowski's test	Present	Absent
8.2	Libbermann-Burchard's test	Present	Absent

Table 3: Total phenolic and flavonoid content of extracts.

Test	Methanolic extract
TPC	69.66 mg/gm equivalent to Gallic acid
TFC	40mg/gm equivalent to Rutin

Table 4: DPPH assay of ascorbic acid and methanolic extract.

S. No.	Conc. (µg/ml)	Ascorbic acid (% Inhibition)	Methanolic Extract (% Inhibition)
1.	**20**	52.03666	42.43094
2.	**40**	57.73931	46.29834
3.	**60**	66.49695	49.39227
4.	**80**	71.89409	53.03867
5.	**100**	85.94705	59.66851
IC 50 Value		**18.99**	**59.21**

Table 5: **Effect of *Pimenta dioica* extract on body weight of the rats.**

Groups	Treatment	Body weight (gms)				
		0 day	**3 day**	**7 day**	**14 day**	**21 day**
Group 1	**Normal control**	204.945± 0.261148	207.1167± 0.236906	211.388± 0.220127	214.121± 0.211115	216.93± 0.251343
Group II	**Alloxan treated Dose (120 mg/kg)**	215.931± 0.2147	208.1883± 0.138982	206.106.± 0.18878	197.1033± 0.250954	180.9217± 0.263381
Group III	**Test 1 P.D Dose- 200mg/kg**	209.0367± 0.223274	208.14± 0.263679	204.375± 0.394316	208.9933± 0.157917	212.9467± 0.163863
Group IV	**Test 2 P.D 400mg/kg**	213.955± 0.255601	210.2983± 0.207773	210.532± 0.138798	216.1367± 0.121234	218.0633± 0.228249
Group V	**Glibenclamide (3 mg/kg)**	217.95± 0.249747	217.0683± 0.271237	221.1883± 0.187944	221.025± 0.204104	225.8383± 0.272683

Table 6: Effect of test samples of extract on blood glucose level in experimental rats.

Groups	Treatment	Blood Glucose Level (gms)				
		0 day	**3 day**	**7 day**	**14 day**	**21 day**
Group 1	**Normal control**	85.075± 0.192523	87.06667± 0.208689	84.385± 0.334083	85.16333± 0.194108	85.075± 0.26996
Group II	**Alloxan treated Dose (120 mg/kg)**	84.19167± 0.1796	274.8533± 0.260316	288.125± 0.238086	295.2167± 0.306536	292.9467 ± 0.228046
Group III	**Test 1 P.D Dose-200mg/kg**	85.095± 0.2263	254.3667± 0.104584	243.8767± 0.496579	219.2233± 0.287711	198.995± 0.093086
Group IV	**Test 2 P.D 400mg/kg**	78.13± 0.095394	271.09± 0.141185	229.9967± 0.201009	182.0717± 0.235449	156.2533± 0.208865
Group V	**Glibenclamide (3 mg/kg)**	76.5233± 0.351033	271.835± 0.264143	226.0217± 0.12142	173.1417± 0.276386	137.2467± 0.342108

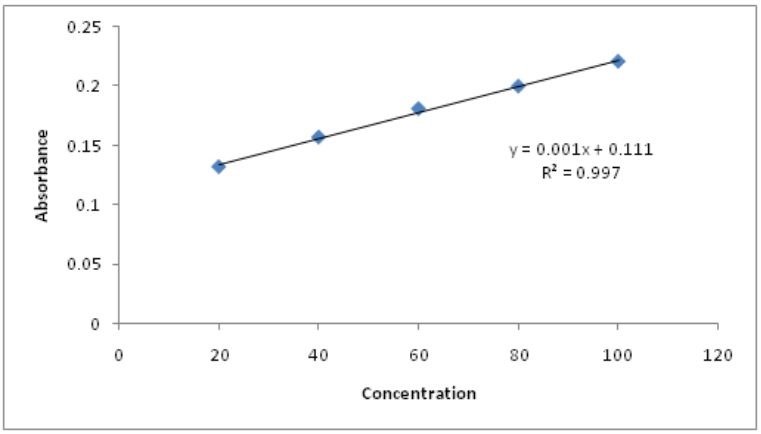

Figure 1: Graph of estimation of total phenolic content.

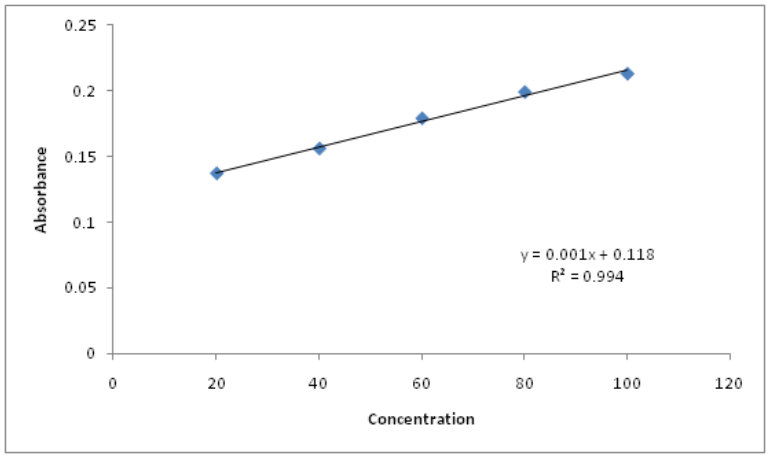

Figure 2: Graph of estimation of total flavonoids content.

CONCLUSION

In order to find and screen the phytochemical components that are essential for the creation of novel medications, medicinal plants are used. Due to the existence of the phytochemical elements, the results of the current study and the prior phytochemical examination are remarkably similar. The total phenolic and flavonoid content in methanolic leaves extract was found to be higher which is further proved by in vitro antioxidant studies. Potential antioxidant activity has good correlations with the therapeutic use in the treatment of cardiovascular disorders. The anti-hyperglycemic properties of *Pimenta dioica* leaves extract were comparable to those of the standard medication used in this investigation; this might be brought about by a rise in insulin production from the pancreatic beta cells that have grown again and a reduction in α-amylase. The presence of bioactive chemicals and the plant extract's ability to produce antioxidants may be responsible for the anti-hyperglycemic potential that has been observed. The outcomes of this research, however, support the plant's conventional use in the management of diabetes.

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