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Hydroethanolic leaf extract of *Parthenium hysterophorus* attenuates blood glucose in alloxan induced diabetic mice.

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Abstract

The *Parthenium hysterophorus* (*P. hysterophorus*) is used to treat diabetes mellitus in local medicinal system of Pakistan but very limited scientifically proved information is available in this context. The objective of this work was to evaluate the antioxidant and antidiabetic activity of *P. hysterophorus* leaf extract. The extraction was made with freeze drying assisted ultrasonication using 40%, 60% and 80% ethanol as solvent. The total phenolic and flavonoid contents were calculated. Antioxidant activity was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and α-glucosidase inhibitory assay was also performed. The *in vivo* hypoglycemic potential of leaf extract was determined in alloxan monohydrate induced diabetic mice. The 60% ethanolic extract exhibited comparatively higher phenolic and flavonoid contents with values of 105.44±2.55 mg GAE/g D.E and 41.50±2.25 mg RE/g D.E, respectively. The 60% extract also possessed lowest IC_{50} value of 87.55 µg/mL and 98.22 µg/mL for DPPH radical scavenging and α-glucosidase inhibition, respectively. The same extract substantially reduced the blood glucose level in alloxan induced diabetic mice and results were quite comparable with standard drug metformin. The extract dose of 450 mg/kg reduced the blood glucose level of diabetic mice from 268.05 mg/dL 137.88 mg/dL at the end of 28 days treatment. The findings confirmed the ethnopharmacological use of *P. hysterophorus* to treat and manage diabetes mellitus type 2. The experimental outcomes may be employed as pharmacological leads to treat diabetes mellitus and to develop functional foods with hypoglycemic attributes.

1. Introduction

The production of reactive oxygen species (ROS) is a fundamental part of metabolism. The abiotic factors are also known to contribute to ROS pool of living system. The excessive production of ROS is harmful to normal cellular functions and biomolecules. The antioxidant defense system of the body maintains the homeostasis by scavenging the ROS (Giacco and Brownlee, 2010). The overproduction of ROS due to any factor results in a state of oxidative stress which is linked to the pathogenesis of many diseases including diabetes. The diabetes is expanding exponentially and type 2 is the major type prevailing in both developing and developed countries. The modern lifestyle, robotic life, consuming high caloric diets and lack of exercise collectively responsible for the development of oxidative stress which further lead to diabetes type 2 (DM-T2) (Vertuani *et al*., 2004). Diabetes may be defined as a metabolic disorder responsible for persistently high blood glucose levels. The DM-T2 is of keen concern to its rapid expansion and associated medical problems. The side complications of diabetes included kidney damage, blindness, cardiac problems, loss of sexual function and many more (Scheen and Paqout, 2013). The synthetic drugs to treat DM-T2 reduce the blood glucose level effectively but are also associated with complex side complications. They lack the ability to reverse the disease towards normal physiology. However, plants are known to possess such dynamic functionalities to treat DM-T2 with improvements in enhanced insulin

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production, regeneration of β-cells and activating adipose tissues to uptake glucose (Yatoo *et al*., 2017). Medicinal plants are the backbone of traditional medicinal systems which are based on believes and observations comprised of hundreds of years (Raza *et al*., 2019). The phytochemicals from plants are well established to counter ROS and consequent health disorders like DM-T2 (Zhang *et al*., 2015). The reduction in ROS and free radicals was responsible for improvements in physiological and hematological indicators, especially during diseased and stressed conditions. The phenolics and flavonoids are metabolites of medicinal significance as most of the therapeutic roles of plants are due to these functional phytochemicals (Seufi *et al*., 2019). The antioxidants and antidiabetic properties of plant phenolics are well established (Moukette *et al*., 2017). Many plants are being used to treat DM-T2 without the scientific confirmations about phytochemicals. The scientifically proved plant species must be used to treat chronic ailments for better outcomes and to avoid toxicity in some cases.

Parthenium hysterophorus (Asteraceae) is known to treat diabetes in many areas of Pakistan (Mahmood *et al*., 2011). *P. hysterophorus* was reported to have important phytochemicals like alkaloids, saponins, tannins and phenolics (Kapoor, 2016). The current work was designed to profile polyphenols and to assess *in-vitro* and *in-vivo* antidiabetic properties to confirm the ethnopharmacological use of *P. hysterophorus* in for DM -T2 management.

2. Materials and methods

2.1 Extraction

The extraction of phytochemicals was performed and the yield extract was calculated. Fresh leaves were treated with liquid nitrogen and ground to a fine powder which was further freeze-dried or lyophilized. The freeze -dried powder material was suspended in 40%, 60% and 80% ethanol for 48 hrs. The extracts were ultrasonicated at 20 kHz for 30 mins and filtered. The extra solvent was removed using a rotary evaporator under vacuum. The extracts were again freeze-dried and stored at -21°C for further use.

2.2 Total phenolic contents (TPC) and total flavonoid contents (TFC)

TPC was determined by following the established method with little modification (Zengin *et al*., 2010). 20 µL of each plant extract was added the 90 µL of Folin-Ciocalteu reagent followed by addition of 91 µL of 10% Na_2CO_3. Absorbance was noted on 726 nm by UV-1700 Schimadzu Japan spectrophotometer. Results were reported as mg gallic acid equivalent per gram dried extract (mg GAE/g D.E).

TFC was determined by using a previously established method based on $AlCl_3$ by Zishen *et al.* (1999). Methanol (2 mL) in 4 mL distilled water and 100 µL extract were mixed with 0.6 mL of 5% $NaNO_2$ solution and 0.6 mL of 10% $AlCl_3$. The resultant mixture was stayed for 10 mins to complete the reaction. Then 4 mL of NaOH (1 molar) and distilled water were added to make a volume of 20 mL and stayed for 25 mins. The absorption of reaction mixture was noted at 510 nm. The results were reported as mg rutin equivalent per gram dried extract (mg RE/g D.E).

2.3 Pre-screening of antioxidant activity by TLC

The thin-layer chromatography (TLC) was used to assess the potential antioxidant activity of plant extracts before going into spectrophotometric assays. The plant extracts were suspended in methanol and 5 µL of this was applied on TLC. The TLC plate was developed by methanol in ethyl acetate (1:1). The developed plate was sprayed by 25% DPPH solution and stayed for 30 mins in dark. The bleaching of the violet color of DPPH by yellow spots was noted (Bektas *et al*., 2005).

2.4 In-vitro antioxidant and anti-diabetic activities

The DPPH radical scavenging by plant extracts was determined to evaluate the antioxidant activity of plant extract. The plant extracts at a concentration of 50-250 µg were used and IC_{50} value was calculated (Chew *et al*., 2011). Simply, 25% methanolic DPPH and plant extracts in concentration (50-250 µg) were dissolved in methanol. Resultant reaction mixture stayed for 35 mins in dark at ambient conditions of temperature and humidity. The absorbance was noted at 517 nm and free radical scavenging was determined in terms of IC_{50} value.

The butylated hydroxyl anisole (BHA) was used as a standard antioxidant compound. The antidiabetic activity was determined by checking the inhibition of α-glucosidase on a spectrophotometer (Jabeen *et al*., 2013). Extracts were dissolved in 70 µL of phosphate buffer (50 Mm) of pH 6.8 and 1 unit/mL of α-glucosidase. Incubation of mixture at 37°C for 10 mins was made and the reaction started upon adding 10 µL of p-nitrophenolglucopyranoside (5mM). After further incubation of 30 mins, absorbance was measured at 405 nm. All the measurements were made in triplicate. Acarbose was used as a standard reference compound and IC_{50} value was computed.

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2.5 In-vivo hypoglycemic activity

The 24 male albino mice (6 weeks old) having an average weight of 31.64±3.05 g were utilized and stayed for a period of 10 days to acclimatize the new environment at animal house of GC University Lahore. After adaptation, the mice were injected with alloxan monohydrate at dose 150 mg/kg body weight. The blood glucose level (BGL) was measured after 72 hrs of injection. The mice having BGL above 200 mg/dL were characterized as diabetic and a control (D0) set of mice was also run (non-diabetic) (Raza *et al*., 2018). The diabetic mice were grouped into untreated mice group (D1), metformin-treated (D2) and extract-treated group (D3). The mice were allowed ad libitum supply of water and food. The extract at a dose of 450 mg/kg body weight was administrated orally in normal saline (Raza, Chaudhary, Mumtaz, Adnan *et al*., 2020). The BGL levels were measured by glucometer by rupturing the vein in the tail every seven days. The monitoring remained active for 28 days.

The standard deviation (±) was applied on the experimental values. The difference of means was computed by applying Analysis of Variance (ANOVA) to see the level of significance in treatment means by using Minitab 17.0 software.

3. Results

3.1 Extract yield, TPC and TFC

The highest extract yield, TPC and TFC were given by 60% ethanol followed by 80% ethanol (Table 1). The 60% ethanolic extract yielded 22.33±0.66%, TPC 105.44±2.55mg GAE/g D.E and TFC 41.50±2.25mg RE/ g D.E. The statistical analysis indicated that the extract yields, TPC and TFC in case of 60% ethanolic extract were significantly higher than the 40% and 80% ethanolic extract ($p<0.05$).

Table 1. Impact of solvent composition on extract yield, TPC and TFC

Extract type	Extract yield %	TPC mg GAE/ g D.E	TFC mg RE/g D.E
40% Ethanolic	19.77±0.52^{b}	88.50±3.45^{c}	33.72±1.13^{b}
60% Ethanolic	22.33±0.66^{a}	105.44±2.55^{a}	41.50±2.25^{a}
80% Ethanolic	20.05±0.38^{b}	92.90±3.57^{b}	35.22±1.53^{b}

Values are expressed as mean±standard deviation. Values with the same superscript within the column are not significantly different.

3.2 Pre-screening of antioxidant activity by TLC

All the extract fractions bleached the violet color of DPPH reagent by yellow spots indicating the qualitative assessment of antioxidant activity by plant extracts.

3.3 In-vitro antioxidant and anti-diabetic activities

The in vitro antioxidant activities were determined by measuring the DPPH free radical scavenging and antidiabetic potential was calculated by observing the α-glucosidase inhibition. The IC_{50} values of extracts, BHA and acarbose for DPPH radical scavenging and α-glucosidase inhibition are given in Table 2. The lowest IC_{50} values for DPPH scavenging and α-glucosidase inhibition were exhibited by 60% ethanol indicating the potential of 60% ethanolic leaf fraction of *P. hysterophorus* to encounter free radicals and to inhibit α-glucosidase activity.

Table 2. The antioxidant and α-glucosidase inhibition by plant extracts

Extracts	IC_{50} values	
	DPPH scavenging IC_{50} value (µg/mL)	α-glucosidase inhibition IC_{50} value (µg/mL)
40% Ethanolic	122.5	133.17
60% Ethanolic	87.55	98.22
80% Ethanolic	105.26	112.85
BHA	85.85	-
Acarbose	-	55.05

3.4 In-vivo hypoglycemic activity

The significant reduction in BGL of diabetic mice was observed in D3 and D2 (Figure 1). The metformin restricted the BGL to 128.12 mg/dl when compared D1 where the final value of BGL was 268.05 mg/dl. The BGL in D3 mice was also reduced gradually and became quite normal by 28 days. The final average value of BGL for D3 mice at the end of 28 days was 137.88 mg/dl. The BGL of D1 mice permanently remained high till the completion of the experiment.

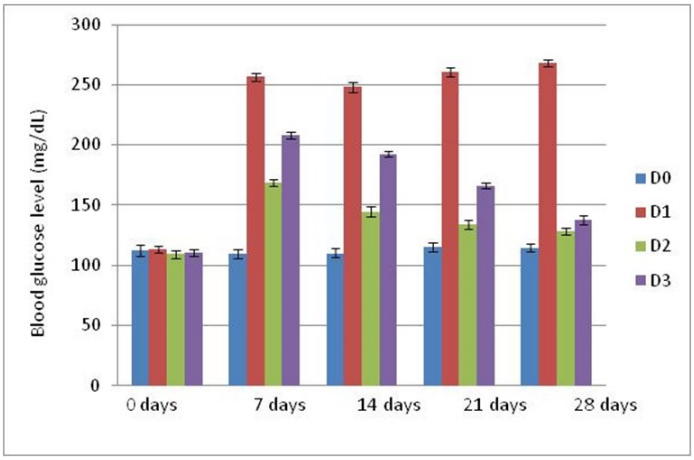

Figure 1. Periodic variation in blood glucose levels of mice under treatment

4. Discussion

The findings revealed that solvent polarity played an

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important role to enhance extract yields. The extract yields also influenced the TPC and TFC values. The high TPC and TFC were of immense importance due to the therapeutic role of phenolic and flavonoids, which are the most common component of plant extracts (William *et al*., 2019). Antioxidant activities of plants are a preliminary indication for their possible hidden medicinal potential (Raza, Chaudhary, Mumtaz, Bashir *et al*., 2020). The antioxidant activity of extracts was assessed by measuring the scavenging of DPPH free radical. The DPPH method is widely adopted and accepted method to evaluate the antioxidant and antiradical activity of substances. The antioxidant activities of hydroethanolic fractions obtained from leaves of *P. hysterophorus* were probably due to the presence of high contents of polyphenols (Arshad *et al*., 2019; Raza *et al*., 2019). The highest antioxidant activity was computed in the case of 60% ethanolic extract likely due to the polarity of solvent composition. A recent study indicated that 60% of ethanol was more efficient to improve the antioxidant activity of *Conocarpus lancifolius* leaf extracts (Raza, Chaudhary, Mumtaz, Adnan *et al*., 2020). The highest DPPH scavenging posed by 60% ethanolic extract was due to high TPC and TFC in the extract. The *in vitro* inhibition of α-glucosidase by extracts was probably associated with phenolic and flavonoid entities. The inhibition of α-glucosidase slows down the breakdown of carbohydrates and delays the glucose absorption in the brush borderline of the intestine. This restriction in absorption results in lowering of postprandial blood glucose level and is strategy is considered as an important tool to manage hyperglycemia (William *et al*., 2018). The structural features of secondary metabolites from plants were reported to bind the specific amino acid residues of enzyme proteins including α-glucosidases. This binding was responsible for the significant reduction in enzyme activity (Shobana *et al*., 2009). A recent study reported that the plant metabolites mainly polyphenols from *Calotropis procera* interacted with amino acid residues of α-glucosidase to inhibit the enzyme activity (Nadeem *et al*., 2019). Another study reported the hypoglycemic potential of *Butea monosperma* which was attributed to phytochemicals in the extract (Farooq *et al*., 2020).

The *in-vivo* trial indicated the potency of *P. hysterophorus* to reduce elevated BGL to great extent and this reduction was quite comparable with the synthetic standard drug metformin. However, it was observed from the findings that metformin reduced the BGL in comparatively rapid fashion as compared to plant extract but at the end of 28 days of the experimental protocol, there was no substantial difference. The reduction in BGL upon administering plant extract was probably due to phytochemicals present in the extract. The most probable mechanism involved in BGL reduction by plant extract was probably the antioxidant nature and α-glucosidase inhibitory properties of phytochemicals in extract (William *et al*., 2019). The synergic effect was proposed for this evident reduction in the glucose of diabetic mice upon consumption of plant extract. The hypoglycemic attributes of plant extract were probably due to many biochemical phenomena at the molecular level including α-glucosidase inhibition, improvement in insulin secretion, regeneration of β cells of the pancreas and enhanced glucose uptake by adipose tissues (Noor *et al*., 2017). The promising *in-vitro* and *in-vivo* antidiabetic potential of 60% ethanolic leaf extract of confirmed the ethnopharmacological use of *P. hysterophorus* to treat diabetes. Moreover, the phytochemical profiling may be expanded to develop novel antidiabetic treatments and functional foods with hypoglycemic properties.

5. Conclusion

The findings of the current work affirmed the ethnopharmacological use of *P. hysterophorus* to reduce the blood glucose concentration to treat diabetes mellitus. The 60% ethanol was the most appropriate solvent for optimum yield of polyphenols. The experimental outcomes of current work provided a potent source of polyphenols in form of *P. hysterophorus* which might be a useful tool for a naturopathic approach towards hyperglycemia. Further studies may be carried out to evaluate the toxicity of *P. hysterophorus* along with identification of effective secondary metabolites of pharmacological importance.

Conflict of interest

The authors declare no conflict of interest.

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