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EXPERIMENTAL PAPER

Antidiabetic and antiplatelet aggregation study of various methanol fractions of *Nymphaea stellata* Willd. leaves

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Summary

Introduction: *Nymphaea stellata* Willd. (*Nymphaeaceae*) is traditionally used for the treatment of diabetes. Alcohol extract of *N. stellata* leaves has been reported for hypoglycaemic activity.

Objective: The aim of this study was to further investigate the different methanol fractions of *N. stellata* leaves for anti-diabetic activity and anti-platelet aggregation activity.

Methods: Methanol extract was fractioned in to unsaponified petroleum ether fraction of methanol extract (UPFME), chloroform fraction of methanol extract (CFME) and residual fraction of methanol extract (RFME). All fractions were evaluated for *in vivo* anti-diabetic activity (STZ-NAD-induced rat model), *in vitro* anti-diabetic activity (PTP1B inhibition study) and anti-platelet aggregation activity.

Results: UPFME showed significant changes in all studied parameters, compared to the diabetic control. UPFME also showed an IC_{50} value of 19.30±1.1 mg/ml and 13.11±0.7 μg/ml in PTP1B inhibition study and anti-platelet aggregation study, respectively.

Conclusion: The study indicates that UPFME of *N. stellata* leaves exhibit anti-diabetic and anti-platelet aggregation activity.

Key words: ***STZ-NAD, PTP1B inhibition, ADP induced, co-TLC, histopathology***

INTRODUCTION

Diabetes mellitus is a metabolic disorder with heterogeneous aetiologies characterized by chronic hyperglycaemia and disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both [1]. The number of people with diabetes has risen from 108 million in 1980 to 422 million in 2014. Type 1 diabetes is characterized by deficient insulin production in the body and type 2 diabetes results from the body's ineffective use of insulin. Type 2 diabetes accounts for the vast majority of people with diabetes around the world [2]. Contemporary anti-diabetic drugs are used only for the management of type 2 diabetes. Hence, there is a need to search for more effective drug for the type 2 diabetes treatment.

Traditional herbal medicines are being prescribed widely in developing countries because of their time-tested effectiveness and relatively low cost. *Galega officinalis* was found to be rich in guanidine, a substance with blood glucose-lowering activity that formed the chemical basis of metformin [3]. Therefore, it would be good to explore the medicinal plants traditionally used for the treatment of diabetes.

Nymphaea stellata Willd. (*Nymphaeaceae*) is commonly known as Indian blue water lily. It is an important and well-known medicinal plant, widely used in Ayurveda and Sidhha systems of medicine for the treatment of diabetes, inflammation, liver disorders, urinary disorders, menorrhagia, blenorrhagia, menstruation problems, aphrodisiac and bitter tonic [4]. Sterols, alkaloids, saponins, tannins and flavonoids are the major class of chemical constituents in *N. stellata*. Oleanolic acid, betulinic acid, gallic acid, β-carotene, lupeol and β-sitosterol has been reported from the methanol extract of leaves [5, 6]. The alcohol extract of *N. stellata* leaves has been reported for hypoglycaemic activity [7]. Hence, the present work was carried out to further investigate the different methanol fractions for potential anti-diabetic activity.

The majority of evidence suggests that inhibiting protein tyrosine phosphatase 1B (PTP1B) represents a highly promising approach in the treatment of diabetes [8]. Platelets play a key role in atherogenesis, and its thrombotic complications and measures, which lead to blockade of one or multiple pathways modulating platelet activation and aggregation processes, are pivotal in reducing ischemic risk in diabetic subjects [9, 10]. Hence, different methanol fractions were also screened for PTP1B inhibition activity and anti-platelet aggregation activity.

MATERIAL AND METHODS

Reagents and solvents

All reagents used were of analytical grade or HPLC grade and were purchased from Sigma-Aldrich, Spectrochem and Merck.

Plant material

Leaves of *N. stellata* were collected on October 2010 from Coonoor and Ootacamund, Tamil Nadu, India. The plant material was identified and authenticated by Dr. A. Rajan, Field Botanist, The Survey of Medicinal Plants and Collection Unit, Government Arts College, Ootacamund, Tamil Nadu, India. A voucher specimen (Pharmacy/HDT/NS/10-11/MKM/07) has been deposited in the Herbarium of Medicinal Plants, Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India.

Preparation of extract and fractions

Air-dried leaves (2000 g) of *N. stellata* were grounded and extracted with methanol in Soxhlet apparatus for 48 h. The extract was concentrated under reduced pressure on a rotary evaporator (Rotavapor, Buchi) and dried in a desiccator to yield methanol extract. The methanol extract was next fractioned by centrifuging with 3 x 500 ml of petroleum ether (60–80°C) at 1000 g for 15 min. The supernatants were combined, concentrated and dried to yield petroleum ether fraction of methanol extract. The residue was subsequently fractioned by centrifuging with 3 x 500 ml of chloroform as mentioned above. The supernatants were combined, concentrated and dried to yield chloroform fraction of methanol extract (CFME). The

insoluble residue was designated as residual fraction of methanol extract (RFME). The petroleum ether fraction of methanol extract was saponified as per the reported procedure [11] and the unsaponified matter was designated as unsaponified petroleum ether fraction of methanol extract (UPFME).

Animals

Healthy adult Albino rats of Wistar strain weighing 200–250 g were procured from Zydus-Cadila Pharmaceuticals, Ahmedabad. The animal house was well ventilated and rats had 12±1 h day and night cycle at 25±3°C and 35–55% relative humidity. Rats were fed with rat pellet feed supplied by Nav-Maharashtra Oil Mills, Maharashtra, India and water *ad libitum*. Animal experiments were carried out as per the guidelines of Institutional Animal Ethical Committee, Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India (IAEC Reg. No. 404/01/a/CPCSEA).

Acute oral toxicity study

Toxicity study was conducted as per internationally accepted protocol drawn under OECD guidelines 423 in Albino rats of Wistar strain. Different groups were administered with UPFME, CFME and RFME at four dose levels (5, 50, 300 and 2000 mg/kg *BW*, p.o.) as a fine suspension in 2% gum acacia. Rats administered with vehicle served as control group.

Anti-diabetic study

Experimental type 2 diabetes was induced as per Masiello *et al.* [12] with streptozotocin-nicotinamide (STZ-NAD). STZ and NAD were procured from Himedia Laboratories Ltd, Mumbai, India. The blood glucose levels were determined at 72 h and the rats with fasting blood glucose concentration of more than 180 mg/dl were considered diabetic and selected for the anti-diabetic study. The selected animals were divided into 9 groups (n=6). Normal rats were administered with 0.5 ml/100 g, *BW* of saline in group I. Diabetic rats were administered with 0.5 m/100 g, *BW* of saline in group II. Diabetic rats were administered with 50 mg/kg, *BW* of metformin [13] in group III. Diabetic rats were administered with 100 and 200 mg/kg, *BW* of UPFME in group IV and V, respectively. Diabetic rats were administered with 100 and 200 mg/kg, *BW* of RFME in group VI and VII, respectively. Diabetic rats were administered with 100 and 200 mg/kg, *BW* of CFME in group VIII and IX respectively. Saline, metformin and fractions were administered orally once a day for 30 days. The effects of different groups were determined by measuring fasting plasma glucose [14], fasting plasma insulin levels [15] and changes in body weight. On 31st day the rats were sacrificed and liver was isolated for the estimation of hexokinase [16], glucose-6-phosphatase [17] and glycogen [18]. Pancreas was also removed to study the histological changes in different groups. Liver and pancreas tissues were washed with normal saline and stored in 10% formalin. The pancreatic tissue was processed for paraffin embedding and sections were stained with haematoxylin-eosin reagent. The histological results were recorded as microphotographs and examined for intracellular changes.

Identification of reported compounds in UPFME by comparative thin layer chromatography (Co-TLC) method

Previously reported compounds like oleanolic acid, betulinic acid, gallic acid, *β*-carotene, lupeol and *β*-sitosterol from methanol extract of leaves [5, 6] were procured from Himedia Laboratories Pvt. Ltd., Mumbai, India and Sigma Chemicals, Bangalore, India. Co-TLC method was preformed with all the reported compounds and UPFME.

PTP1B inhibition study

PTP1B inhibitory activity was tested by using Calbiochem® PTP1B colorimetric assay kit (User Protocol; 2008, Catalogue No: 539736, USA). The absorbance was measured at 620 nm on microplate ELISA reader (BioRad-680XR) and the results were expressed as the amount of phosphate released in nM.

Anti-platelet aggregation study

In vitro ADP induced platelet aggregation activity was carried out using heparin-treated whole blood obtained from healthy anaesthetized rats by electrical impedance method using Chrono-Log Model 592VS dual channel whole blood aggregometer (Chrono-Log Corporation, Haverton, PA, USA) [19]. Each reading was taken in triplicate with

different concentrations of samples, taking control and the respective concentration of aspirin for comparative reading each time. The 50% inhibition of platelet aggregation was determined for each test sample comparing with the control and IC_{50} values was calculated accordingly in μM for standard and μg/ml for fractions.

Statistical analysis

The quantitative measurements in all the experiments were made on 6 rats in each group and the values are expressed as mean ± standard deviation. Graphpad Instat Version 4 software was used. Data were subjected to the analysis of variance (one way ANOVA) to determine the significance of changes followed by Dunnett's test for multiple comparisons.

RESULTS AND DISCUSSION

Percentage yield of extracts and fractions

The percentage yield of methanol extract, petroleum ether fraction of methanol extract, UPFME, CFME and RFME were found to be 22.44, 19.17, 17.63, 11.23 and 69.04% *w/w*, respectively.

Acute oral toxicity study

The acute toxicity study was performed for establishing the therapeutic index. The fractions showed no signs of toxicity up to a dose of 2000 mg/kg.

Anti-diabetic study

The diabetic control group showed a marked increase in plasma glucose and a reduction in insulin level. UPFME 200 mg/kg and metformin treated group showed significant ($p<0.01$) restoration of glucose and insulin levels when compared to the diabetic control group. CFME and RFME treated groups at both dose levels did not show any significant change (tab. 1).

It has been suggested that bioactive compounds from plant sources having anti-hyperglycaemic activities might act by several mechanisms such as stimulation of insulin secretion, increasing the repair or proliferation of β-cells and enhancing the effects of insulin [20]. Hence, the significant decrease in the glucose level and increase in insulin level of diabetic rats treated with UPFME may be due to the stimulation of insulin secretion from the remnant β-cells or regenerated β-cells or both [21].

Metformin and UPFME 200 mg/kg treated groups showed a significant ($p<0.01$) increase in hexokinase level (tab. 2). The activation of glycolysis may be the reason for the significant increase of hexokinase in UPFME 200 mg/kg treated rats.

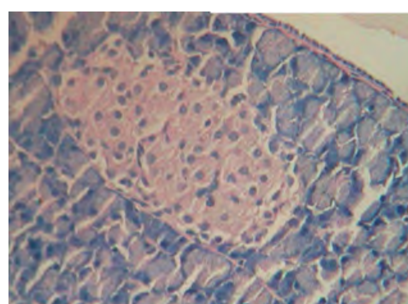
Metformin and UPFME 200 mg/kg treated groups showed significant ($p<0.01$) decrease in glucose-6-phosphatase level (tab. 2). The decreased levels of glucose-6-phosphatase observed in UPFME treated diabetic rats may be due to the regulation of gluconeogenic enzymes.

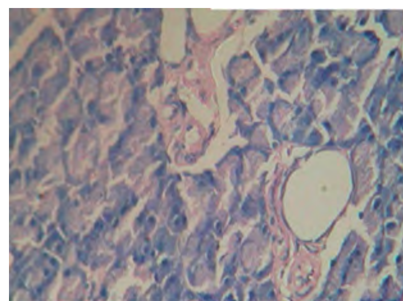
The hepatic glycogen content of diabetic control group was reduced significantly as compared to other groups. Metformin and UPFME (100 mg/kg and 200 mg/kg) treated groups showed significant ($p<0.01$) restoration of the depleted glycogen level than other groups (tab. 2). The prevention of liver glycogen depletion in UPFME treated groups may be due to stimulation of insulin release from β-cells.

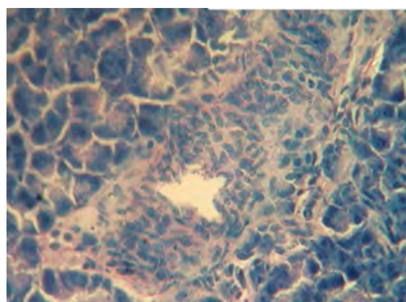
STZ-NAD induced diabetes is associated with a characteristic loss of body weight which is due to increased muscle wasting in diabetic state [22]. Metformin and UPFME 200 mg/kg treated groups showed significant ($p<0.01$) restoration of body weight (tab. 3). The improved carbohydrate and lipid metabolism may be the reason for increased body weight.

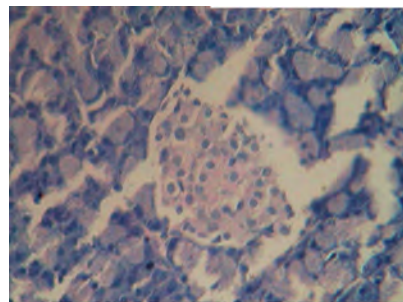
Diabetic control group showed shrunken islets of Langerhans with disrupted cellular architecture and disarray of acinar structure. Significant reduction in total number of cells per pancreatic islet with marked degranulation was also observed. This provides clear evidence that the pancreatic β-cells have been destroyed in diabetic control group (fig. 1b) when compared to the intact β-cells in normal control group (fig. 1a). The metformin treated group showed significant higher number of cells per islet with its cellular architecture preserved. It also showed no degranulation and vacuoles (fig. 1c).

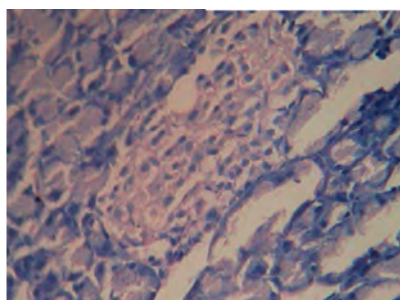
UPFME (100 mg/kg) treated (fig. 1d), CFME (100 and 200 mg/kg) treated (fig. 1f and 1g) and RFME (100 and 200 mg/kg) treated groups (fig. 1h and 1i) showed shrunken islet with disrupted cellular architecture. They also showed significant reduction in total number of cells per islet and more vacuoles. UPFME (200 mg/kg) treated groups showed relatively intact, larger size islet and reduced vacuoles (fig. 1e). Significant increase in number of cells per islet in UPFME (200 mg/kg) treated group suggests regeneration of pancreatic islet cells.

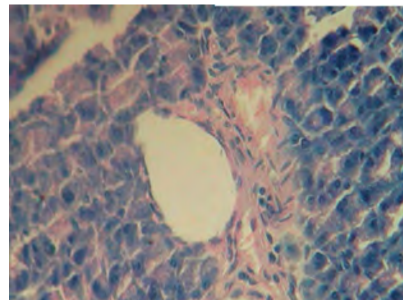

(a) Normal control group

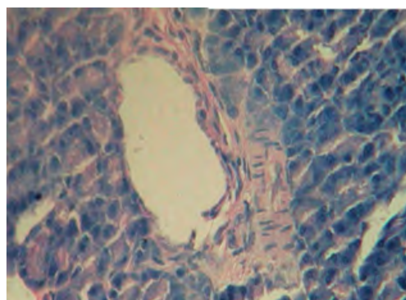

(b) Diabetic control group

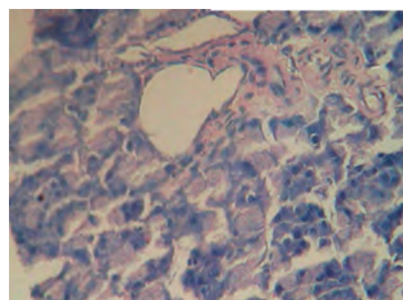

(c) Metformin treated group


(d) UPFME (100 mg/kg) treated group


(e) UPFME (200 mg/kg) treated group


(f) CFME (100 mg/kg) treated group


(g) CFME (200 mg/kg) treated group


(h) RFME (100 mg/kg) treated group

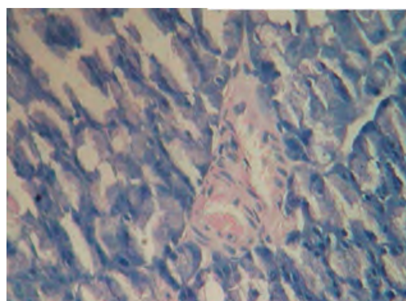

(i) RFME (200 mg/kg) treated group

Figure 1.

Histological studies showing changes in the endocrine and exocrine pancreas of normal, diabetic, standard and *Nymphaea stellata* fractions treated groups

Table 1.
Plasma glucose and insulin level changes in normal, diabetic, standard and *Nymphaea stellata* fractions treated groups

Groups	Plasma glucose [mg/dl]	Plasma insulin [μU/ml]
I (Normal control)	87.04±4.86 **	16.59±2.02 **
II (Diabetic control)	275.34±28.86	4.86±1.05
III (Metformin 50 mg/kg)	145.26±24.22 **	14.56±1.54 **
IV (UPFME 100 mg/kg)	240.54±14.34 *	7.01±0.89 *
V (UPFME 200 mg/kg)	190.57±21.57 **	11.58±1.07 **
VI (CFME 100 mg/kg)	262.59±12.19 ^{ns}	6.47±0.87 ^{ns}
VII (CFME 200 mg/kg)	250.67±13.51 ^{ns}	6.01±1.47 ^{ns}
VIII (RFME 100 mg/kg)	265.49±10.53 ^{ns}	5.16±1.27 ^{ns}
IX (RFME 200 mg/kg)	255.31±15.11 ^{ns}	5.94±1.01 ^{ns}

Value are means ± standard deviation (n=6); * – $p<0.05$; ** – $p<0.01$; ^{ns} – not significant.

Table 2.
Changes in hepatic hexokinase, glucose-6-phosphatase and liver glycogen levels in normal, diabetic, standard and *Nymphaea stellata* fractions treated groups

Groups	Hexokinase [U/g/min]	Glucose-6-phosphatase [U/g/min]	Liver glycogen [μg of glucose/mg of wet tissue]
I (Normal control)	10.51±1.97 **	14.82±2.57 **	53.78±2.18 **
II (Diabetic control)	2.24±1.69	35.38±6.54	25.13±3.21
III (Metformin 50 mg/kg)	8.54±1.1 **	19.35±5.67 **	52.24±1.28 **
IV (UPFME 100 mg/kg)	3.12±0.85 ^{ns}	26.15±4.57 *	34.15±3.31 **
V (UPFME 200 mg/kg)	5.67±0.48 **	23.68±3.58 **	40.11±2.12 **
VI (CFME 100 mg/kg)	2.75±1.12 ^{ns}	33.65±5.47 ^{ns}	27.94±4.11 ^{ns}
VII (CFME 200 mg/kg)	3.12±1.87 ^{ns}	30.27±3.69 ^{ns}	28.12±3.21 ^{ns}
VIII (RFME 100 mg/kg)	2.98±1.68 ^{ns}	34.65±4.19 ^{ns}	28.04±5.27 ^{ns}
IX (RFME 200 mg/kg)	3.15±0.81 ^{ns}	29.68±3.31 ^{ns}	29.11±5.61 ^{ns}

Value are means ± standard deviation (n=6); * – $p<0.05$; ** – $p<0.01$; ^{ns} – not significant.

Table 3.
Body weight changes in normal, diabetic, standard and *Nymphaea stellata* fractions treated groups

Groups	Body weight [g]
I (Normal control)	226.13±4.57**
II (Diabetic control)	165.24±9.56
III (Metformin 50 mg/kg)	220.35±4.87 **
IV (UPFME 100 mg/kg)	174.84±5.66 ^{ns}
V (UPFME 200 mg/kg)	190.22±5.12 **
VI (CFME 100 mg/kg)	170.35±7.11 ^{ns}
VII (CFME 200 mg/kg)	171.94±4.59 ^{ns}
VIII (RFME 100 mg/kg)	168.22±3.67 ^{ns}
IX (RFME 200 mg/kg)	171.41±6.66 ^{ns}

Value are means ± standard deviation (n=6); ** – $p<0.01$; ^{ns} – not significant.

The overall results show that CFME and RFME at both dose levels (100 mg/kg and 200 mg/kg) are completely inactive. UPFME has shown nearly comparable effect to that of metformin on all the studied parameters.

Identification of reported compounds in UPFME by comparative thin layer chromatography (Co-TLC) method

The Co-TLC studies were performed between the compounds viz. oleanolic acid, betulinic acid, gallic acid, *β*-carotene, lupeol and β-sitosterol with UPFME separately in different mobile phases. The presences of reported compounds were confirmed by comparing their R_f values with UPFME (tab. 4).

Anti-hyperglycaemic effect of oleanolic acid [23], β-carotene [24], lupeol [25] and β-sitosterol [26] may have contributed synergistically to the observed anti-diabetic activity of UPFME. Increased body weight observed in UPFME treated rats may also be due to the antidyslipidemic activity of betulinic acid [27] and lupeol [25]. The signs of β-cell regeneration in the histopatholgical study may also be due to the antioxidant effect of β-carotene and/or gallic acid present in UPFME. From the results, it may also be postulated that at least more than one constituent with diversified mechanism of actions may be the reason for the total anti-diabetic activity of UPFME.

PTP1B inhibition study

Suramin is a reversible and competitive inhibitor of PTP1B. UPFME showed the highest potency of PTP1B inhibition with an IC_{50} value of 19.30±1.1 µg/ml among the other fractions (tab. 5). Oleanolic acid [28], betulinic acid [29] and lupeol [30] has been previously reported for PTP1B inhibition activity. The activity of UPFME may be due to the synergistic action of oleanolic acid, betulinic acid and lupeol. The *in vitro* PTP1B inhibition of UPFME overlaps with its *in vivo* anti-diabetic activity.

Anti-platelet aggregation study

Aspirin and UPFME inhibited platelet aggregation with an IC_{50} value of 10.14±0.7 µM and 13.11±0.7 µg/ml respectively (tab. 6). The exhibited activity of UPFME can be attributed to the anti-platelet aggregation activity of oleanolic acid [31] and gallic acid [32]. CFME and RFME increased aggregation of platelets.

CONCLUSION

The anti-diabetic activity (*in vivo* and *in vitro*) and anti-platelet aggregation activity of UPFME from

Table 4.
Identification of reported compounds in UPFME by co-TLC method

Samples/standards	R_f	Mobile phase	Derivatisation agent
UPFME	0.53	Toluene-ethyl acetate-glacial acetic acid (7:3:0.1 *v/v/v*)	Anisaldehyde sulphuric acid reagent
Oleanolic acid	0.53		
UPFME	0.56	Toluene-ethyl acetate-glacial acetic acid (7:3:0.03 *v/v/v*)	Anisaldehyde sulphuric acid reagent
Betulinic acid	0.56		
UPFME	0.26	Toluene-ethyl acetate-methanol-formic acid (6:3:1:0.5 *v/v/v/v*)	Visible in short UV (254 nm)
Gallic acid	0.26		
UPFME	0.39	Hexane-benzene (9:1 *v/v*)	Visible in normal light
β-Carotene	0.39		
UPFME	0.40	Toluene-chloroform-ethyl acetate-glacial acetic acid (10:2:1:0.03 *v/v/v/v*)	Antimony trichloride reagent
Lupeol	0.40		
UPFME	0.57	Toluene-chloroform-methanol (4:4:1 *v/v/v*)	Anisaldehyde sulphuric acid reagent
β-Sitosterol	0.57		

Table 5.
PTP1B inhibitory effect of standard and fractions of *Nymphaea stellata*

Standard/fractions	IC_{50}
Suramin	5.37±0.6 µM
UPFME	19.30±1.1 µg/ml
CFME	46.33±1.2 µg/ml
RFME	95.11±1.4 µg/ml

Value are means ± standard deviation (n=3).

Table 6.
Effects of standard and fractions of *Nymphaea stellata* on ADP induced platelet aggregation

Standard/fractions	IC_{50}
Aspirin	10.14±0.7 µM
UPFME	13.11±0.7 µg/ml
CFME	Induced aggregation
RFME	Induced aggregation

Value are means ± standard deviation (n=3).

N. stellata leaves is due to its synergistic multi-target effect. The currently marketed drugs for type 2 diabetes are based on the so-called "one-molecule-one-target" paradigm. However, due to the multi-factorial pathogenesis of the disease, drugs that hit more than one biological target may offer a better pharmacological approach.

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Conflict of interest: Authors declare no conflict of interest.

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Studium działania przeciwcukrzycowego i antyagregacyjnego różnych frakcji wyciągu metanolowego z liści *Nymphaea stellata* Willd.

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Streszczenie

Wstęp: *Nymphaea stellata* Willd. (*Nymphaceae*) jest tradycyjnie stosowana w leczeniu cukrzycy. Istnieją doniesienia o działaniu hipoglikemicznym wyciągu alkoholowego z liści tej rośliny.

Cel: Celem pracy były dalsze badania właściwości przeciwcukrzycowych i antyagregacyjnych różnych frakcji metanolowego wyciągu z liści *N. stellata.*

Metodyka: Ekstrakt metanolowy frakcjonowano na niezmydloną frakcję otrzymaną za pomocą eteru naftowego (UMFME), frakcję otrzymaną za pomocą chloroformu (CFME) oraz pozostałość ekstraktu metanolowego (RFME). Wszystkie frakcję były badane w kierunku działania przeciwcukrzycowego *in vivo* (w modelu szczurzym cukrzycy wywołanej przy pomocy STZ-NAD), w kierunku aktywności przeciwcukrzycowej w badaniach *in vitro* (badania hamowania PTP1B) oraz w celu określenia aktywności antyagregacyjnej.

Wyniki: Stosowanie frakcji UMFME powodowało znaczące zmiany we wszystkich badanych parametrach zwierząt doświadczalnych (w porównaniu do grupy kontrolnej z wywołaną cukrzycą). Wartość IC_{50} dla frakcji UMFME wynosiła 19,30±1,1 µg/ml w badaniu hamowania PTP1B oraz 13,11±0,7 µg/ml w badaniu działania antyagregacyjnego.

Wnioski: Otrzymane wyniki badań wskazują, że frakcja UMFME z liści *N. stellata* wykazuje aktywność przeciwcukrzycową i antyagregacyjną.

Słowa kluczowe: ***STZ-NAD, hamowanie PTP1B, indukcja ADP, metoda co-TLC, histopatologia***