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Evaluation of antidiabetic activity, metabolic profiling and determination of major metabolites by LC-ESI/MS/MS of Novobet

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Abstract
Diabetes mellitus is a heterogeneous disease developing under the result of the congenital combination and acquired factors, unhealthy lifestyles, especially wrong eating habits, hypokinesis and characterized by chronic progressive course leading to severe micro and macrovascular complications. *Geranium collinum*, *Glycyrrhizaglabra*, and *Rhuscoriaria*, are species growing in Tajikistan, and are characterized as natural sources of phenolic compounds that have several known biological activities and serve as a traditional medicine against various disorders including type 2 diabetic diseases. The chemical profile of "Novobet" was analyzed by LC-ESI-MS/MS. "Novobet" was screened for *in vitro* antidiabetic (PTP-1B enzyme inhibition) and antioxidant effects (DPPH radical scavenging). The *in vivo* antidiabetic and acute toxicity of "Novobet" was evaluated in animal models. Based on the pH of the medium, the new anti-diabetic phytomedicine, namely "Novobet", was developed. Glucose and lipids levels and the concentration of malondialdehyde (MDA) in serum of laboratory animals treated with "Novobet" were investigated. The blood sugar level in experimental rabbits, after treatment with 5mL/kg of "Novobet", was decreased after 7, 15 and 30 days, respectively, which indicated active hypoglycemic action. In addition, the level of total lipids in the blood decreased to 44.5%, and the level of blood cholesterol under the influence of the "Novobet" decreased to 32.2%. The MDA level of rabbit's blood, after treatment with "Novobet" at the dose of 5mL/kg, decreased significantly. A total of 25 major compounds were identified in "Novobet" by LC-ESI-MS/MS spectroscopy. The new Phytomedicine "Novobet", exhibited potent antidiabetic and antioxidant activity and therefore it can serve as a natural antidiabetic and antioxidant nutraceutical.

Keywords: *diabetics;* geranium collinum; glycyrrhizaglabra; rhuscoriaria; *alloxanhydrate; PTP-1B*

1. Introduction
Diabetes mellitus is a group of metabolic diseases described by hyperglycemia resulting from defects in insulin secretion and insulin action or both. The role of free radicals in diabetes has been widely discussed, and the involvement of free radicals in diabetes onset and complications has been shown experimentally [1]. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. Hyperglycemia is the distinguishing feature of diabetes, and persistent conditions in a diabetic patient lead to the formation of oxidative stress for multiple reasons, including the autoxidation of glucose [2]. Chronic low-grade inflammation in T2DM has given an impetus to the field of immunometabolism linking inflammation to insulin resistance and β-cell dysfunction [3]. Many factors advocate a causal link between metabolic stress and inflammation. Numerous cellular factors trigger inflammatory signaling cascades, and as the result, T2DM is at the moment considered an inflammatory disorder triggered by disordered metabolism [4]. Cellular mechanisms like activation of Toll-like receptors, endoplasmic reticulum stress, and inflammasome activation are related to the nutrient excess linking pathogenesis and progression of T2DM with inflammation [5].
The scientific research confirms that the uric acid has an important role in the development of diabetes [6], and cardiovascular pathology [7]. It is also known that the increased level of uric acid aggravates the form of acidosis in an organism, contributes to the production of endogenous alloxan, which has a diabetogenic effect [6]. The great scientist-doctor Avicenna has written one thousand years ago regarding the existence of "unnatural matter",

formed in the organism of patients with diabetes [8]. According to Avicenna research, patients have a change "mizadzh" from a neutral side, characterized for a healthy state, to the "cold" side, which corresponds to the development of metabolic acidosis [4]. Therefore, it is important to apply the method of treatment "opposite to the opposite", to give the balance of "mizadzh", i.e. neutralization of diabetes-related form of acidosis [4].

Geranium collinum Stephan ex Willd.is a perennial plant that belongs to the family Geraniaceae. In Tajikistan there are more than 20 species, which belong to three genera, namely *Pelargonium* L' Hér., *Geranium* L. and *Erodium* L' Hér. *G. collinum* is one of the most common plants in Central Asia [9]. In Tajikistan, it grows in the wild areas of Zarafshan, Varzob and Hissar valley, Darwaz, Western Pamir at an altitude of 800–2700 m above sea level. *G. collinum* has been used for the treatment of rheumatism, gout, dysentery, external and internal bleeding, as well as in the treatment of skin wounds, eczema, scabies, tenosynovitis and pruritus [10], and *in vitro* antidiabetic effects of several extracts were evaluated [9].

Glycyrrhizaglabra L. (licorice) is an herb that has medicinal value and has been used since ancient times as a sweetener and a remedy for a great diversity of ailments. Roots of *Gl. glabra* contain a high concentration of the saponin glycyrrhizin, which is the main sweet-tasting component [11]. Glycyrrhizin and glycyrrhetinic acid are the major active constituents obtained from licorice roots, one of the most widely used herbal preparations for the treatment of liver complaints. The plant also used as an anti-inflammatory, spasmolytic, laxative, anti-depressive, anti-ulcer and anti-diabetic [12]. The hot decoction prepared from the roots of *Gl. glabra* is known in Tajik and Uzbek traditional medicines for its therapeutic effects against nervous disorders, flatulence and other digestive disorders [13].

Rhuscoriaria L. is a shrub known as “sumac”, belonging to the Anacardiaceae. *R. coriaria* is traditionally used as a table spice particularly with rich dishes and is highly recommended for adjustment of the blood lipids in diabetic patients. It is used as an herbal remedy in traditional medicine owing to its anti-fibrogenic, antifungal, anti-inflammatory, antimalarial, antimicrobial, antimutagenic, antioxidant, antithrombin, antitumorigenic, antiviral, cytotoxic, hypoglycaemic, leukopaenic and atheroprotective effects [14].

This investigation is related to the preparation of the new antidiabetic phytomedicine, "Novobet", which is made up from the roots of *G. collinum* and *Gl. glabra*, and the fruit of *R. coriaria* growing in Tajikistan. The objective of the present study was to evaluate of the *in vitro* and *in vivo* antidiabetic activity of different combinations of the three medicinal plants and to chemically characterize the major chemical components by LC-ESI/MS/MS.

2. Materials and methods

2.1 General Procedures

Ultrapure water was generated by a Millipore water purification system (EMD Millipore, Billerica, MA, USA). All reagents and chemicals were of analytical grade.

2.2 *In vitro* evaluation of antidiabetic and antioxidant activities: PTP-1B enzymatic and DPPH assays

The enzyme activity was measured using the PTP-1B ((3-(3, 5-dibromo-4-hydroxy-benzoyl)-2-ethyl-benzofuran-6-sulfonicacid-(4-(thiazol-2-ylsulfamyl)-phenyl)-amide) enzyme inhibition assay according to the literature [9, 15]. Antioxidant activity was measured using the DPPH (1, 1-diphenyl-2-picrylhydrazyl) scavenging assay procedure as published in the literature [16].

2.3 Animals

The "Novobet" was tested for anti-diabetic, hypolipidemic and antioxidant activities in 80 mature rabbits of both sexes with 1.8 – 2.5 kg weight. Acute toxicity of "Novobet" was studied on 108 mature white mice (18-20 g) and 108 white rats (180-210 g). Permission for the study was obtained from the country government. Animals were purchased from the Toxic-pharmacological Laboratory of Avicenna Tajik State Medical University.

2.4 Preparation of diabetic rabbits, white mice and white rats

Alloxan diabetes was induced by subcutaneous injection of the freshly prepared 10% solution of alloxan hydrate at a dose of 80 mg/kg for 13-15 hours in rabbits with average weights of 1.8-2.5 kg. The experiments were carried out on 108 mature white mice with an average mass of 18-20 g, as well as 108 non-native white rats of both sexes with an average mass of 180-210 g. The rats were allowed to acclimatize to the laboratory condition for several days. Each animal was used once only in all of experiments.

2.5 Plant materials and preparation of "Novobet" decoction

Underground parts of *G. collinum*, *Gl. glabra*, and fruit of *R. coriaria* were collected from Hushyori Valley in the Republic of Tajikistan in September 2016. The roots and fruit were authenticated by Professor Yusuf Nuraliev and voucher samples have been deposited in the herbarium of the Institute of Avicenna’s Medicine and Pharmacology, Academy of the Tajik Traditional Medicine. Dried and ground roots and fruit of plants 1000 g (*G. collinum* 650 g, *Gl. glabra* 100 g and *R. coriaria* 250 g) with the ratio of (1:10) were submitted to extraction with 10000 mL of distilled water at 90°. The concentration was 27.8 mg/mL. The 5ml (139 mg) of "Novobet" was used to evaluate antidiabetic and antioxidant activities in rabbits. In order to study acute toxicity, the volume of freshly prepared decoction "Novobet" in the experiments was reduced by 10 times using evaporation in a water bath. The final concentration of "Novobet" decoction was 278 mg/mL, respectively. The obtained "Novobet" aqueous decoction was stored at 4°C until use.

2.6 Experimental design

In the present experiment, 80 rabbits and 108 mature white mice and 108 white rats of both sexes were used. The animals (rabbits) were divided into 4 groups. Twenty rabbits were used in each group.

Group1: Intact group.

Group 2: Negative control group (untreated), rabbits were injected with alloxan hydrate after 18 hours of fasting; distilled water was administered by IG, based on 5 ml/kg body weight, daily during the 30-day test period.

Group3: Experimental group, rabbits, 30 minutes prior to injection of alloxan hydrate, "Novobet" (5 ml/kg of body weight) and subsequently during 30 days, the decoction of "Novobet" was injected daily into the intact fetus at a dose of 5 ml/kg of body weight.

Group 4: Positive control group, experimental animals following the same schedule, before and after injection of alloxan hydrate, an infusion (1:10) of "arfazetine" was administered by IG daily at a dose of of 5 ml/kg of body

weight.

2.7 Biochemical parameters, peripheral and coagulation analyses and acute toxicity determination

The blood was collected by retro-orbital puncture, and the biochemical parameters (hemoglobin and blood coagulation) were evaluated. In order to determine the safety level of the collection test in accordance with the Pharmacological Committee requirements of the Health Ministry of the Republic of Tajikistan, acute toxicity of the "Novobet" broth on white mice and white rats were studied. A total of 108 mature white mice and 108 white rats of both sexes were divided into nine groups with animals 6 per group. The mice fasted for 12 h with access to water. To determine the acute toxicity of the drug during parenteral administration, the "Novobet" decoction was administered orally to white mice at doses of 0.5, 1, 2, 3, 4, 5, 6.5, 7, and 8 ml/kg body weight, and administered subcutaneously to white rats at the rate of 0.5, 1, 1.5, 2.5, 3, 3.5, 4.5, 5, 6.5 ml/kg body weight. A single dose preparation more than 1 ml/kg of the mass was fractionally administered orally and subcutaneously at 0.5 ml with 30 minutes intervals [17]. Observation of the experimental animals was carried out for the first 48 hours, and also for the next 14 days after a single drug administration. The activity and appearance of the animals were monitored. The changes were recorded in time to determine LD_{50} and LD_{100}.

2.8. LC-MS/MS analysis

LC system coupled to a linear ion trap mass spectrometer (4000 Q TRAP) from AB Sciex equipped with column oven, a solvent delivery pump and an auto-sampler. Analyst 1.5 software was used to control LC-ESI-MS. The chromatographic separation was performed on a reversed stationary phase column (XBridge™ C18, particle size 5 µm, 4.6×150 mm with guard column). The column temperature was maintained at 35°C. Gradient elution was carried out using mobile phase A: Methanol and B: 2% formic acid in water. The column temperature was kept as ambient and a flow rate of 1 µl per minute was used. Zero time condition was 10% A and a linear gradient to 60% A was applied up to 40 minutes. Then a linear gradient to 100% A up to 55 minutes was applied and the column was equilibrated at 100% A for 5 minutes. The whole analysis took 65 minutes. MS was used in negative mode and the scanning was performed in the mass range m/z values from 100 to 2000. Ten microliters (10 µL) of a sample was injected into the chromatographic column.

2.9 Statistical analyses

The data obtained in the morphological analysis and caspase-3 quantification tests were analyzed using one-way ANOVA with Dunnet' spost-test ($p<0.05$) for comparison with the control using the Bioestat 5 software.

3. Results & Discussion

3.1 Optimization of "Novobet" extract according pH medium

The optimal composition and ingredient proportions of the herbal collection for new anti-diabetic Phytomedicine "Novobet", were developed on the basis medicinal plants growing in Tajikistan. Initially, six proportions of plants were prepared in process of development for "Novobet" to determine the pH of the medium. Changing of pH medium of decoction samples of "Novobet" was measured by using pH indicator (Fig 1).

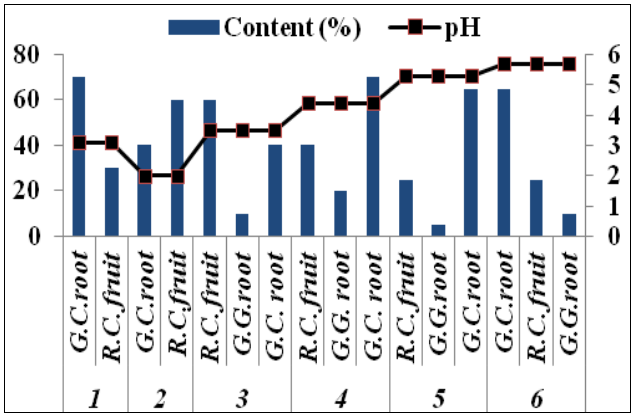

Fig 1: The pH medium of "Novobet" depends on components percentage**.** Note: G.C. - *G. collinum; R.C. - R. coriaria; G.G.-Gl. glabra*

Figure 1 shows the pH of the prepared decoctions of "Novobet", the acidity generally depends on the percentage of *R. coriaria* fruit in the mixture. In all cases when the contents of the *R. coriaria* fruit were above 30% (compositions 1, 2 and 3), a sharp decline in pH was observed. However, the addition of *Gl. glabra* root up to 20% did not significantly change the pH to neutral or alkaline. When the amount of the *R. coriaria* fruit was reduced to 25% along with increasing percentage of the *Gl. glabra* root up to 5% and 10% resulted in freshly prepared decoctions with pH closer to neutral. Based on the results of the experiments, we considered it appropriate to develop the final version of the new anti-diabetic collection of "Novobet" according to the following proportions of active ingredients: powdered roots of the *G. collinum* (65%), roots of *Gl. glabra* (10%) and fruit of *R. coriaria* (25%). Thus, the above ratio resulted in a "Novobet" with a slightly acidic pH of 5.8, comparable to the infusion of "arfazetin" (1:10) with the pH=5.7.

3.2 PTP-1B inhibition screening and DPPH radical assay

The effect of the prepared "Novobet" was determined for their *in vitro* evaluation of percent inhibition of the enzyme PTP-1B and DPPH free radical scavenging activity. The significant antidiabetic activity of "Novobet" was evaluated with an IC_{50} 0.88 µg·mL^{-1} which more than value of positive control (PTP-1B Inhibitor) with IC_{50} 1.46 µg·mL^{-1}. The free radical scavenging activity of the "Novobet" was measured *in vitro* by 2,2-diphenyl-2-picrylhydrazyl (DPPH) assay. A lower IC_{50} value implied a better antioxidant activity of the tested sample. "Novobet" showed potent antiradical activities with IC_{50} value of 6.26 µg·mL^{-1}. Vitamin C was used as the positive control and tests were carried out in triplicate. The IC_{50} value of Vitamin C was 5.34 µg·mL^{-1}.

3.3 *In vivo* evaluation of the antidiabetic and antioxidant effect of "Novobet" on diabetic rabbits

The main criteria for the anti-diabetic properties of "Novobet" were survival of animals from diabetes, glucose and lipids levels, and the concentration of MDA in serum. The blood sugar content was determined in each series before taking the drug and injection of 80 mg/kg b. wt with alloxanhydrate, in 7, 15 and 30 days after daily administration of the test substances [18]. Fasting blood glucose was estimated after induction to determine the severity of glucose elevation among the induced groups. The levels of lipids and MDA in blood were determined in 18-20 hours after 30 days oral intake of "Novobet" and "arfazetine" infusion. "Arfazetine" is

a packaged herbal tea, which has a regulating effect on carbohydrate metabolism (hypoglycemic effect) [19, 20].

Subcutaneous injection of freshly prepared 10% solution of alloxanhydrate with the dose of 80 mg/kg in rabbits, fasting for 13 - 15 hours already showing clinical symptoms of diabetes for 7 days – polyuria, polydipsia (thirst), hyperglycemia and hyperlipidemia were mentioned. According to the results of the 30-day-treatment course with the "Novobet", the blood sugar level in experimental rabbits, after the application of "Novobet", decreased in comparison with 7, 15 and 30 days of the untreated series by 7.1, 5.8 and 5.3 mmol/l, respectively, that indicated active hypoglycemic action of the new agent (Figure 2A).

The level of total lipids in the blood decreased to 44.5% (7.1 compared to 12.8 mmol/l) with the untreated series. The level of blood cholesterol under the influence of the "Novobet" significantly decreased to 32.2% (2.3 mmol/l) with untreated series (control 217.7%) 3.4 mmol/l. The content of triglycerides in treated animals that were treated by "Novobet" noticeably decreased to 42.2% (1.7 mmol/l), compared to the untreated series, 110% (2.5 mmol/l). As a result of a monthly treatment course with "Novobet", the level of blood phospholipids, which are considered to be the main anti-atherogenic factors, increased to 61.5% (1.05 mmol/l) in relation to the corresponding parameters with control animals 0.65 mmol/l (Figure 2B).

The results of the total lipid profile showed that alloxanhydrate injection led to the development of hyperlipidemia in which serum triglyceride and total cholesterol increased significantly ($P < 0.05$) when compared to the non-diabetic rats in group 1. The antioxidant action of "Novobet", as measured by the content of MDA in the blood, was also determined after 18-20 hours after the month-long treatment with the herbal remedies.

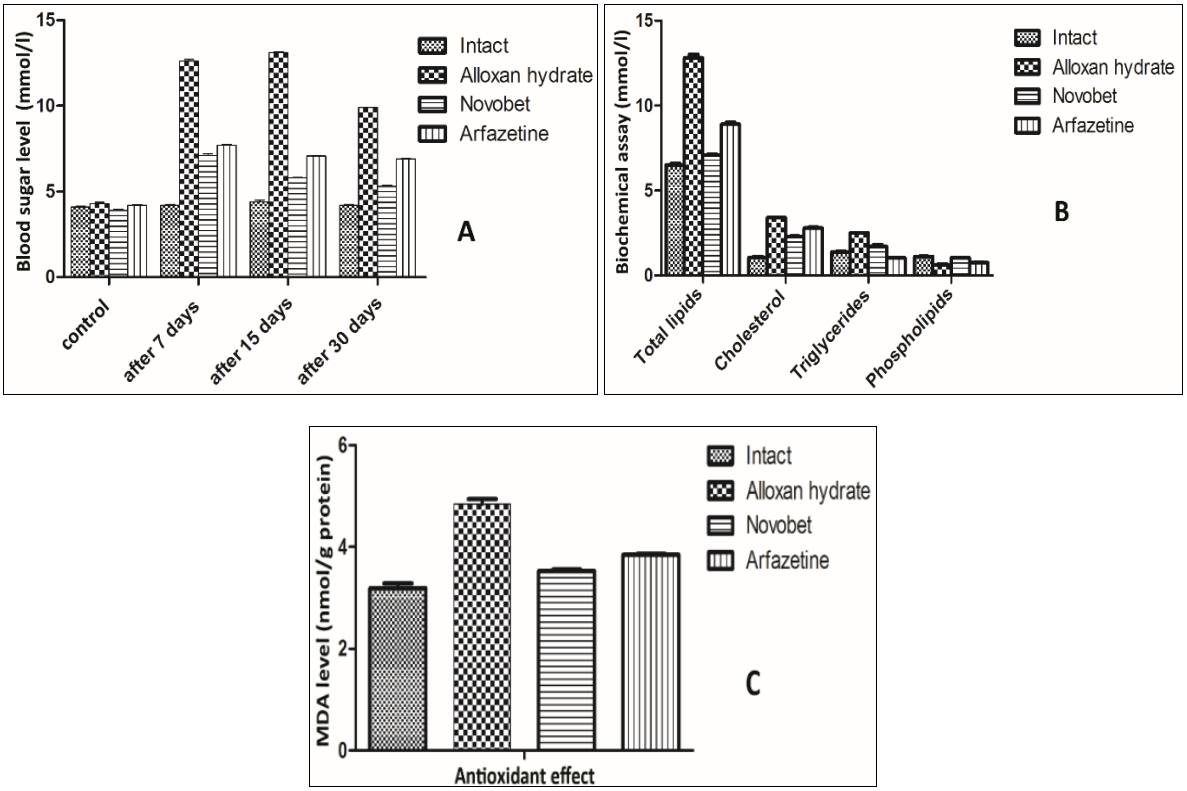

Fig 2: Comparative sugar-lowering, hypolipidemic and antioxidant effect of "Novobet" decoction (1:10) and "arfazetine" infusion (1:10) with alloxanhydrate diabetes; hypoglycemic action - at the different study times (A); hypolipidemic action at the end of treatment course (B); antioxidant effect at the end of treatment course (C).

As a result of the 30-day treatment with the "Novobet" in a dose of 5 ml/kg, by increasing alloxan hydrate diabetes, the MDA level of rabbit's blood decreased by 35.9% (3.53 nmol/g proteins), compared with the control series at 52 % (4.85 nmol/g proteins). Antioxidant activity of "Novobet" manifested by 15.3% more than the "arfazetin" infusion (Figure 2C). The percentage of survival rate in animals treated during one month with "Novobet" was 12 (60%), and in rabbits treated with "arfazetine" infusion, the index was equal to 9(45.7%). "Novobet" exhibited an excellent antioxidant activity. "Novobet" is enriched in polyphenols, suggesting the antioxidant and antidiabetic activities of medicinal plants. The plant content is enriched polyphenols can lower the risk of these diseases [21]. Inhibition of PTP-1B activity can decrease absorption of the carbohydrates and effectively reduce postprandial blood glucose to achieve the goal of blood glucose control. We observed that *G. collinum* had a strong activity in the inhibition of PTP-1B enzyme [9].

3.4 Acute toxicity of "Novobet" in various laboratory animals

Each dose of "Novobet", orally and subcutaneously, was examined on 8 white mice, and also on 6 white rats. Observation of the experimental animals was carried out within 48 hours and after 14 days after the drug administration. With oral administration of the "Novobet" in doses of 1 and 2 ml/kg of mass, no visible changes were observed in the behavior of the experimental white mice and rats, except for mild depression. Only after intragastric administration of drug in doses exceeding 3 ml/kg of weight in experimental animals, oppression of general behavior, a sharp decrease in motor activity occurred. The clinical result of the overall large doses effect of "Novobet" in mice and rats was similar and generally characterized by mild depression of

the nervous system, especially adynamia. Within 30-40 minutes the animals refused to eat food and water. The depression state in white mice and rats after intragastric administration of the drug in doses above 4 ml/kg of the body mass was continued for 18-20 hours. Death of mice with intragastric administration of decoction in doses exceeding 5, 6 ml/kg mass was observed on days 1-3 and, more rarely on days 5 from the beginning of the drug administration. Only with oral drug administration in doses of 5 and 6 ml/kg of body weight, death of single experimental white mice and white rats was occurred. A dose of 7 ml/kg of mass, when administered orally, was lethal to 50% LD_{50} of white mice. Absolutely lethal for white mice with intragastric administration was a dose of 8 ml/kg of body weight. White rats were less resistant to the toxic properties of the "Novobet". Single deaths for white rats occurred after oral administration of the drug at doses of 5-6 ml/kg of body weight. The dose of 7 ml/kg of mass during administered orally was the LD_{50}. "Novobet" in a dose of 8 ml/kg of mass caused the death of all experimental white rats, i.e., this dose was absolutely lethal LD_{100}.

3.5 Toxicity of "Novobet" decoction for white mice and white rats with subcutaneous drug administration

With the hypodermic introduction of "Novobet", the drug toxicity increased significantly. The pattern of acute poisoning by the drug was similar to the toxic effect observed for oral administration. During the first 5-10 minutes, the motor reaction in all experimental white mice and white rats was markedly increased. Respiration sharply increased, reaching 140-180 respiratory movements per minute against 115-120 respiratory events in the control animals. Then there was a state of nervous system oppression, a general adynamia with a sharp slowdown in motor activity. Treated animals became sluggish, gathered in one heap, refused active intake of food and water. Against the background of general adynamia, especially in experimental white mice, the reaction of animals to sound and mechanical stimuli sharply increased. After 1.5-2 hours from the beginning of parenteral large doses administration of "Novobet", a noticeable skin blanching (especially in auricles region) and visible mucous membranes were observed. Mostly the death of animals in a state of general adynamia often occurred in white mice and rats. The deaths caused by the large doses of "Novobet" generally accompanied recurring clinical attacks of limb spasms. A dose of 6.5 ml/kg of mass with subcutaneous administration was the LD_{50}, and a dose of 7.5 ml/kg of mass was found to be absolutely fatal for white mice. The average lethal dose of "Novobet" decoction for white rats with subcutaneous injection was found to be 6.5 ml/kg weight, i.e. In general, it was almost 30% higher than LD_{50} drug for white mice. "Novobet" in a dose of 7.5 ml/kg of weight with subcutaneous administration caused the death of all 8-tested rats and thus this dose was an absolutely lethal dose LD_{100} of the drug. Based on the toxicological studies on two types of laboratory animals (white mice and white rats), we conclude that a "Novobet" decoction to be minimally toxic.

3.6 The overall state of peripheral blood and coagulation system in animals that were treated for six months with "Novobet"

We have studied the effect of different drug doses the overall state of the peripheral blood in experimental animals that received different doses of "Novobet" for 6 months. The experiments were carried out on 53 mongrel white rats with an average mass of 190.0-230.0 g, which were injected inside/stomach for six months. "Novobet" was tested in doses of 2 and 5 ml/kg of weight. The control series animals were injected with distilled water at a rate of 5 ml/kg mass weight. Blood was obtained by lower gum dissection for hematologic analysis.

The results of these experiments indicated that the studied doses of "Novobet" with prolonged administration did not cause significant changes in the condition of the peripheral blood. After six-month daily administration of the drug at a dose of 5 ml/kg weight in both control and experimental animals, some statistically inaccurate data ($P > 0.05$) were within physiological fluctuations. By using "Novobet" in a dose of 2 ml/kg mass, the hemo-stimulating effect of the drug was stronger than drug administration in a dose of 5 ml/kg weight. By using this dose, the leucostimulating effect of the drug was clearly marked, which manifested itself in a significant (by 19.2%) and statistically significant ($P < 0.05$) total increase in leukocyte number. There was also an active increase in the number of stab neutrophils (by 42.8% at $P < 0.01$) and monocytes (by 44.9% at $P < 0.001$). The ESR parameters in animals that received "Novobet" for 2 months at doses of 2 and 5 ml/kg of mass were identical in all cases with the intact series animals' data, also indicating that the drug was not hematotoxic.

The substances contained in "Novobet", with a single and especially repeated administration, are able to mediate the blood coagulation process. Therefore, to assess the degree of drug safety, a depiction of the blood coagulation process was determined at the end of the six-month experiment in both control and experimental animals that received different "Novobet" doses daily. For this purpose, in control and experimental animals, blood coagulation time (sec.), prothrombin time (sec.), prothrombin index (%), recalcification time (sec.), fibrinogen (g/l), fibrin (mg %) and thrombotest was determined (Table 1).

After the six-month intragastric administration of "Novobet" in doses of 2 ml/kg, the amount of coagulation time was increased by 124.6% ($P<0.05$). The maximum effect of coagulation time increase (by 130.7%) was observed using "Novobet" in a dose of 5 ml/kg mass. Blood coagulation time reduction in experimental rats that received different doses of "Novobet" within 6 months is evidence of an easy increased in total blood coagulation capacity under the influence of new phytopreparation studied. It was established experimentally that, in intact white rats, blood coagulation occurs in an average of 65.0 ± 1.2 sec (Table 2).

Table 1: The peripheral blood results of white rats that received "Novobet" under the chronic (6 months) experimental conditions in different doses. The average of 8-12 cases in each series.

Parameters	Experiment series and doses in ml/kg mass		
	Intact	"Novobet" decoction 2 ml/kg	"Novobet" decoction 5 ml/kg
Hemoglobin g/l	144.0 ± 0.4	$\frac{161.6 \pm 0.2}{P > 0.05}$	137.2 ± 0.6
Erythrocyte x 10^{12}/l	4.98 ± 0.06	$\frac{5.0 \pm 0.1}{P > 0.1}$	4.96 ± 0.1

ESR, mm/h	3.3 ± 0.04	3,4 ± 0,3	2.8 ± 0.03
Leukocyte x 10^3/l	5.2 ± 0.03	$\frac{6.2 \pm 0.03}{P < 0.05}$	5.12 ± 0.01
Neutrophils: Stab (nuclear stick)	2.8 ± 0.03	$\frac{4.0 \pm 0.1}{P < 0.01}$	3.2 ± 0.03
segment-nuclear	49.0 ± 0.1	$\frac{47.8 \pm 0.07}{P > 0.05}$	50.8 ± 0.09
Eosinophils	3.8 ± 0.09	$\frac{4.6 \pm 0.07}{P < 0.05}$	3.8 ± 0.03
Basophiles	0.3 ± 0.01	0	0
Lymphocytes, %	3.8 ± 0.1	36,0 ± 0,1	37.4 ± 0.07
Monocytes, %	4.83 ± 0.03	$\frac{7.0 \pm 0.1}{P < 0.001}$	5.2 ± 0.03

Note: The value of P for the experimental series is given in comparison with the corresponding parameters of intact group animals

Table 2: Antidiabetic influence of "Novobet" to coagulation time in white rats under chronic experiment conditions.

Experiment series and doses in ml/kg mass	The blood coagulation time	
	in seconds	% increase
1. Intact – distilled water, 5 ml/kg	65,0 ± 1,2	Accepted as 100 %
2. "Novobet" decoction, 2 ml/kg	81,0 ± 1,5	124,6%
3. "Novobet" decoction - 5 ml/kg	85,0 ± 0,8	130,7%

Figure 3 displayed the results of six-month intragastric administration of the "Novobet" in doses of 2 and 5 ml/kg mass which did not cause any significant changes in coagulation system (prothrombin time, prothrombin index, recalcification time, fibrinogen, etc.) parameters. In control and experimental animals, the observed slight deviations in all cases were within physiological fluctuations.

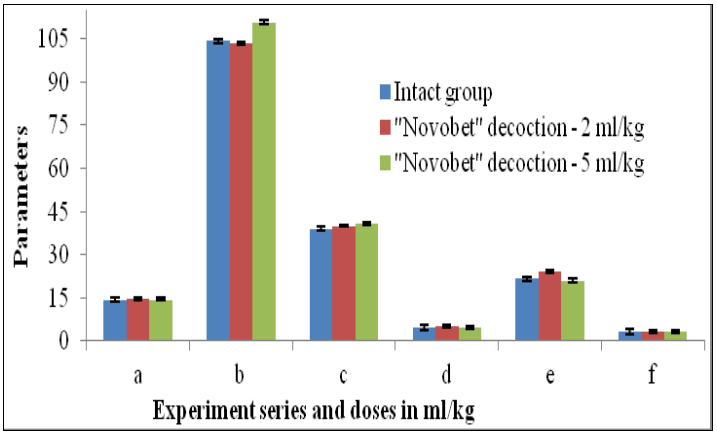

Fig 3: Parameters of blood coagulation system in white rats that received "Novobet" decoction in chronic experiment conditions (6 months).
Note: a-prothrombin time, sec.; b- prothrombin index, %; c-recalcification time, sec.; d- fibrinogen, g/l; e- -fibrin, mg,; f- trombotest, level

The increase in blood coagulation time is not accompanied by a noticeable increase in other parameters that characterize the first and second coagulation processes. Therefore, this effect cannot be considered to be dangerous for diabetics. Thus, the results obtained showed an absence of hemotoxic, as well as homeostasotoxic effects in pharmacological properties of the components in the antidiabetic phytopharmaceutical formulation "Novobet".

3.7 Effects of "Novobet" decoction on the urinary kidney function in a chronic experiment

Table 3 summarizes the effects of "Novobet" on the urinary kidney function in a chronic experiment that animals were treated in a dose of 2 ml/kg. The maximum increasing diuresis (5.19 ± 0.1 ml) occurred after 2 hours from the beginning of the water load. Under the action of this dose, a 20% (P<0.05) increase in urine excretion was observed during 4 hours compared with the control series.

In experimental animals that received "Novobet" with a dose of 5 ml/kg of weight in a chronic experiment, the diuresis process increased up to 40.8% in 4 hours compared with the control series. The maximum volume of excretory urine (4.8 ± 0.06 ml) was observed 1 hour after the water load. Thus, in experimental animals that received "Novobet" over 6 months, the release of excess fluid from the body occurred after 1 (dose 5 ml/kg) and 2 hours (dose 2 ml/kg) from the beginning of the water load. This effect has great practical value and helps to promote not only excretion of excess liquid, but also from many different metabolic, as well as xenobiotic metabolites.

In the control animals, the urine reaction was acidic in all cases with a pH of 6.6. In contrast, in the experimental group of rats that were treated with different doses of "Novobet", moderate alkalization (pH was 7.7) of urine reaction was observed. This effect is very positive and indicates an active endo-cleansing (of various metabolic) action of "Novobet", which is important not only for diabetic patients but also for people suffering from uratosis.

Table 3: Urinary function of the kidney in white rats that received different doses of "Novobet" decoction for 3 months.

Experiments series dose in ml/kg mass	Time in hours	Diuresis data after water load	
		Average urine volume in ml	pH of urine
1. Control, distilled water – 5 ml/kg	1	2.68 ± 0.1	6.6 ± 0.1
	2	3.47 ± 0.8	
	3	2.85 ± 0.03	
	4	1.3 ± 0.06	
	total 4 h	10.3 ± 0.09	
		100%	
2. "Novobet" decoction - 2 ml/kg	1	2.36 ± 0.04	7.7 ± 0.03
	2	5.19 ± 0.1	
	3	2.74 ± 0.06	
	4	2.02 ± 0.03	
	total 4 h	12.37 ± 0.23	
		20%* P<0,05**	
3. "Novobet" decoction - 5 ml/kg	1	4.8 ± 0.06	7.7 ± 0.02
	2	3.82 ± 0.05	
	3	3.12 ± 0.06	
	4	2.81 ± 0.02	
	total 4 h	14.5 ± 0.19	
		40.8% P<0.01	

Note: * - in percentage with relation to the control series; ** - the P value is given in comparison with the control series.

3.8 LC-ESI-MS/MS analysis

Application of LC-ESI-MS/MS technique in the current investigation provided useful information to characterize of 25 phenolic compounds in the decoction (1:10) of "Novobet". The mixture of crude 70% ethanol extract of "Novobet" was analyzed by LC-MS/MS which was operated in negative and positive ionization modes. The experiment was performed in triplicate, delivering the signals for different compounds at various m/z values. Figure 4 shows the total ion chromatogram obtained from LC-MS analysis of the Novobet.

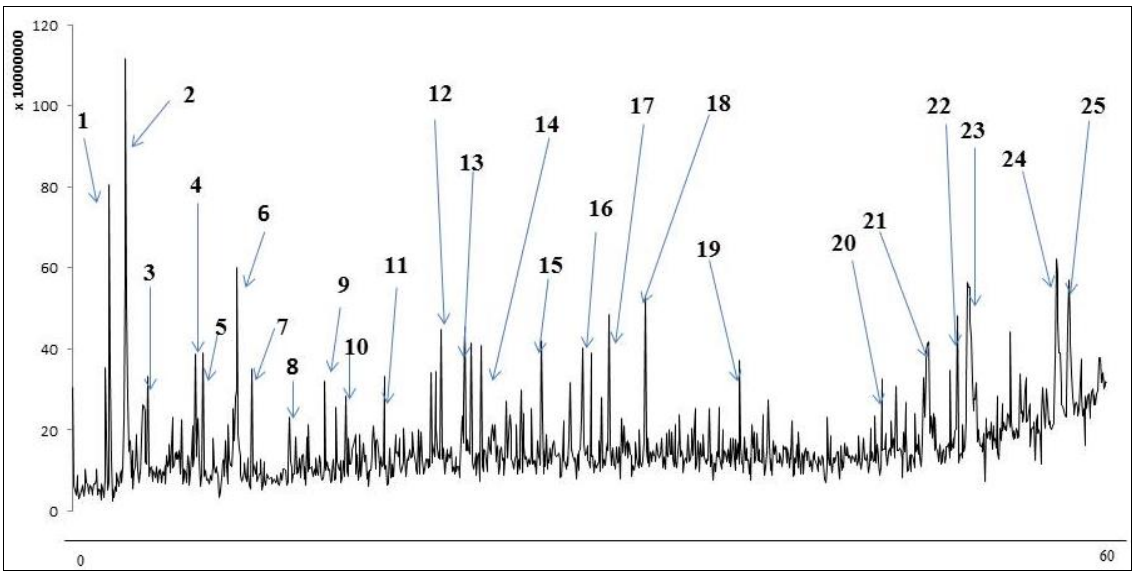

Fig 4: LC MS/MS total ion chromatogram of the "Novobet".

All the major compounds identified through LC-MS/MS analysis along with their peak retention times, fragmentation patterns and the references on the basis of which they have been identified, are summarized in Table 4.

Table 4 show that the decoction (1:10) of the "Novobet" consists mainly of phenolic compounds, when compared with the literature data for the identified compounds.

Table 4 shows the result LC-ESI-MS-MS analysis of "Novobet". Total of 25 compounds have been identified by comparing their fragmentation behavior with the published literature. Compounds name, their retention time, m/z value of molecular ion peak in negative ionization mode, fragmentation pattern and the references against which the peaks appeared have been assigned the compounds have been tabulated in table 4. Fragmentation pattern has not been discussed as all the compounds identified in the understudied extract are known compounds and have been discussed in the already published literature in detail. Several individual compounds, namely 3,3′,4,4′-tetra-*O*-methylellagic acid, 3,3′-di-*O*-methylellagic acid, quercetin, caffeic acid, flavonols (+)-catechin, (–) - epicatechin, (–) -epigallocatechin, gallic acid, β-sitosterol-3-*O*-β-D-glucopyranoside, and corilagin were

previously isolated in pure form from *G. collinum* by [9]. Quantification analysis of the major components of *G. collinum* were determined via UHPLC DAD detector [10]. Antidiabetic actions of flavanols might contribute to prevention or delay in T2DM onset by modulating insulin secretion in pancreatic cells and targeting insulin-sensitive tissues because of their insulin-like activity or through the regulation of key proteins of the insulin-signaling pathways. Flavanols have been proved to enhance glucose uptake through the promotion of glucose transport, to repress glucose production, or to improve lipid metabolism [22].

Table 4: LC/ESI-MS-MS data of the major compounds detected from "Novobet" (negative mode).

No	RT	Compounds name	$[M-H]^-$	Fragmentation	References
1	2.16	caffeic acid	178.7	178.9; 161.2;	[23]
2	3.20	caffeic acid hexoside	341	340.8; 179.0	[23]
3	4.26	galloyl-hexoside	331	331.1 313,0 271.0; 211.1	[24,25]
4	7.22	gallic acid	169	169.0; 153.4	[23]
5	7.44	quercetin derivative	594.2	408.0; 289.1; 246.0	[26,27]
6	9.40	3-o-galloyl quinic acid	343	191, 169,	[28]
7	10.41	trans-cinnamic acid	147	147.2	[29]
8	12.66	epicatechin	289	289.1	[30]
9	14.96	digalloylquinic acid	495	343; 191; 169	[28]
10	15.97	protocatechuic acid	151.9	152.0	[25]
11	18.21	catechin	289	289.1	[30]
12	21.42	eriodictyol	289	289.0	[30]
13	22.85	digalloyl-hexoside	483	331.0; 313.5; 169.1	[31]
14	23.84	p-coumaric acid	162.2	162.2; 119.0	[23]
15	27.51	trigalloyl-hexosidei	635	483.1; 465.1; 169.1	[31]
16	29.90	Catechins hexoside	451	451.1; 313.1; 289.2; 109.4	[30]
17	31.47	quercetin	301	301.1	[30]
18	33.42	caffeoyl-6'-secologanoside	551	551.2; 389.2;341.2	[30]
19	39.15	ellagic acid	301	257;229;185	[28]
20	47.03	kaempferol	285	285.0	[30]
21	50.14	isorhamnetin	315	315.2	[30]
22	51.55	3-o-methylellagic acid 4'-o-β-d-arabinopyranoside	447	315; 300	[28]
23	52.32	ginsenoside Ro	438	393.6; 249.5; 203,6; 191,	[26,27]
24	57.52	chlorogenic acid	353	352.9; 191.2; 179.2	[23]
25	57.88	glycyrrhizic acid	821.5	351.0; 803.1; 778.3;	[32]

4. Conclusions

The prolonged effect study of "Novobet" formulation in rodent models under chronic experimental conditions revealed no shifts of urine acidic reaction side. This confirms the chemical-physical stability, pharmacological appropriateness and compatibility of the newly created formulation of components. The manifestation of active hypoglycemic, hypolipidemic and antioxidant action of "Novobet" decoction allows us to recommend this remedy, not only for the treatment of insulin-independent T2DM of mild and moderate severity but also for the therapy of the other pathologies which pathogenesis is associated with hyperlipidemia, including hypercholesterolemia, as well as related to the increased level of lipid peroxidation products. Results of the present investigation suggest that diabetes is associated with an increase in oxidative stress as shown by the increase in serum malondialdehyde (MDA) and also, diabetes is associated with an increase in serum total cholesterol, phospholipids as well as triglycerides levels. Therefore, we conclude that "Novobet" is a potential source of bioactive compounds with a significant antidiabetic effect *in vitro* and *in vivo* and that the identified formulation can be considered a potential antidiabetic and antioxidant agent with promising effects in the treatment of T2DM and oxidative stress. Our investigations suggest the development of herbal formulations of a new antidiabetic drug based on the roots of *G. collinum*, *Gl. Glabra* and fruits of *R. coriaria* growing in Tajikistan.

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Author Contributions

S.N., M.S. performed the phytochemical investigation, designed and wrote the manuscript; F.S., L.J., G.L. analyzed dates; M.S., M.H., studied the bio-pharmacological activities; W.N.S., F.S., S.N. made a critical revision of the manuscript.

Conflicts of Interest

Conflicts of Interest: The authors declare no conflict of interest.

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