ANK-1, ANK-2, ITL-2 Polyphenols in a Dexamethasone-Induced Rat Model of Type 2 Diabetes Mellitus Treatment

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Abstract

Background: Diabetes mellitus (DM) is a prevalent endocrine disorder that significantly increases the risk of cardiovascular complications due to the dysfunction of the coagulation and anticoagulation systems, exacerbated by metabolic imbalances. Despite advancements in understanding DM's pathophysiology, the precise alterations in the hemostatic system remain inadequately explored, posing challenges in diagnosis and treatment. This study investigated the effects of polyphenolic compounds isolated from Hexagalloy-lglucose (ANK-1), Hepta galloyl-glucose (ANK-2), and Isatis tinctoria L. (ITL-2) on biochemical and coagulation parameters in a dexamethasone-induced type 2 diabetes mellitus (T2DM) model in rats. Methods: T2DM was induced in 25 aged white outbred rats by administering dexamethasone (0.150 mg/kg) twice daily, with a control group receiving saline. Biochemical parameters, including glucose, total protein, ALT, AST, cholesterol, and triglycerides, were measured using a semi-automatic analyzer. Coagulation parameters such as prothrombin time (PT), activated partial thromboplastin time (APTT), plasma recalcification time (RP), and fibrinogen levels were assessed using a single-channel coagulometer. Platelet aggregation was recorded using an aggregometer, and clot degradation was measured spectrophotometrically. Results: Dexamethasone administration induced significant hyperglycemia and hypercoagulation, evidenced by reduced PT, APTT, and RP, alongside increased fibrinogen levels. The administration of ITL-2 normalized all biochemical parameters, while ANK-1 and ANK-2 normalized glucose, cholesterol, and triglycerides but not ALT and AST levels. ANK-1, ANK-2, and ITL-2 demonstrated significant antithrombotic effects, with ITL-2 showing the highest efficacy, reducing clot mass and enhancing clot degradation. Conclusion: Polyphenolic compounds, particularly ITL-2, exhibit potential as therapeutic agents for managing coagulation abnormalities and hyperglycemia in T2DM. These findings highlight the importance of further research into plant-derived substances as safer and effective alternatives in diabetes management.

Keywords: Polyphenols, Type 2 Diabetes Mellitus, Hemostasis, Dexamethasone, Hypercoagulation, Anticoagulants, Antiplatelet agents, Polyphenols.

Significance | This study demonstrated the potential of specific polyphenolic compounds in regulating hemostasis and biochemical imbalances in type 2 diabetes mellitus.

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Introduction

Diabetes mellitus, the most prevalent endocrine disorder, is increasingly associated with serious vascular complications. This

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condition is marked by disruptions in the balance between coagulation and anticoagulation systems, which exacerbate circulatory system dysfunction due to metabolic disturbances (Ametov & Solov'eva, 2007). Research by the European Society of Cardiology and the European Association for the Study of Diabetes highlights a significant rise in cardiovascular complications among diabetic patients, including coronary heart disease and ischemic stroke, which more than doubles the risk compared to non-diabetic individuals (Atamanov, Yakovleva, & Tereshchenko, 2003).

Diabetes mellitus is classified under the VIII group of hematogenous thrombophilias, characterized by disturbances in various hemostatic system components due to metabolic changes and altered blood composition (Atamanov et al., 2003). Studies have shown that diabetes leads to abnormalities in hemocoagulation and fibrinolysis, with increased fibrinogen levels, shortened thrombus formation times, and reduced fibrinolytic activity as the disease progresses (Carr, 2001; Brummel & Mann, 2002). Additionally, there is a positive correlation between blood fibrinogen levels and glycemia (Kretova, Kondratyeva, & Sukhanova, 2004).

Despite established trends toward thrombosis in type 2 diabetes mellitus, the precise mechanisms underlying changes in hemostatic parameters remain incompletely understood, complicating laboratory diagnosis and treatment (Kirichuk, Bolotova, & Nikolaeva, 2004). As the number of type 2 diabetes patients continues to rise, there is an ongoing need for novel antidiabetic drugs with enhanced therapeutic efficacy and safety profiles. Plant-derived compounds, which have shown promise in preventing the progression of diabetes and its cardiovascular complications, are of particular interest. Our previous screening of over fifty biologically active substances identified several polyphenolic compounds with significant activity.

The aim of this study is to investigate the effects of polyphenolic drugs, including Hexagalloyl-glucose (ANK-1), Heptagalloyl-glucose (ANK-2), and Isatis tinctoria L. (ITL-2), on biochemical parameters and blood coagulation in a dexamethasone-induced type 2 diabetes model in rats.

2. Materials and Methods

Type 2 diabetes mellitus was experimentally induced in 25 adult white outbred rats (18 months old, initial body weight 300-350 g) fed a high-fat diet. The diabetes model was established by subcutaneously administering dexamethasone (Dexamethason Ukraine) at a dose of 0.150 mg/kg twice daily to 15 rats. A control group of 5 rats received a saline solution (0.3 ml). On the third day of treatment, blood samples were collected from the conjunctiva of the rats' eyes into epindorph tubes. To assess antithrombotic activity, 50 µl of blood was used, and for coagulation studies, 0.5 ml of blood mixed with citrate (1:9) was centrifuged at 3000 g for 10 minutes.

Biochemical parameters including glucose, total protein, alanine aminotransferase (ALAT), aspartate aminotransferase (AST), total cholesterol, and triglycerides were measured in blood serum using a semi-automatic biochemical analyzer (Rayto Life and Analytical Sciences Co., Ltd) with test kits from Cypress Diagnostica, Belgium.

Coagulogram parameters such as prothrombin time (PT), activated partial thromboplastin time (APTT), plasma recalcification (RP), and fibrinogen (F) were evaluated using a single-channel coagulometer (CYANCoag, Belgium, CY003, SN:5400439) (Луговской, 2003). Platelet aggregation was measured with the Born method using a Biola ALAT-2 aggregometer (No. FSR2007/01301, Russia), with ADP (5-10 µmol), adrenaline, collagen, and ristomycin as aggregation inducers (Lee & Bae, 2015). Aggregation results were expressed as a percentage of the maximum light transmission (T%, max), with aggregation curves automatically calculated by a computer interfaced with the aggregometer.

To induce acute hyperglycemia, rats were fasted, weighed, and their baseline blood glucose levels were determined. Subsequently, they were divided into groups of 10 and hyperglycemia was induced via a single intragastric injection of a hypertonic glucose solution (5000 mg/kg). Blood glucose levels were measured one hour post-injection using the glucose oxidase method.

For antithrombotic activity assessment, fresh blood clots (50 µl) were mixed with 0.5 ml of saline solution and incubated at 37°C for one hour. The antithrombotic activity was evaluated by measuring color adsorption of the supernatant at 410 nm using a spectrophotometer (AA = color of the experimental supernatant / color of the control supernatant). The clot density was determined by weighing the re-formed clot, and clot inhibition or induction (CI) was calculated using the formula: SIS (%) = (mass of clot in control – mass of clot in experiment) / (mass of clot in control) × 100.

Polyphenolic compounds (ANK-1 from Hexagalloy-lglucose, C48H36O30, molecular weight 1092; ANK-2 from Heptagalloyl-glucose, C55H40O34, molecular weight 1244; and ITL-2 from Isatis tinctoria) were administered orally starting on the third day of the experiment at a dose of 5 mg/kg for five days. The control group received the same volume of saline solution (0.5 ml). The effects of these polyphenolic compounds on platelet ADP aggregation were examined in vitro using rat platelet-rich plasma and the Biola ALAT-2 aggregometer.

ANK-1 is a pale yellow amorphous powder, Rf 0.42 (1-system), liquid. h. 298-301 0C (with decay). UB spectrum (MeOH, λmax, nm): 230, va 279. Mass spectrum ESI-MS negative analysis, m/z 1092 [M-H]-, MS/MS-decomposition products 939, 787, 769, 635,

617, 483, 465, 447, 431, 331, 295, 169. 1H PMR spectrum (CD3OD), glucose ring: δ 6.28 (d, 8.3 Hz, H-1), 5.58 (d, 8.3 Hz, H-2), 5.98 (m, 9.6 Hz, H-3), 5.62 (m, 9.6 Hz, H-4), 5.58 (dd, 9.6, 8.3 Hz, H2), 4.54 (m, H-5), 4.45 m (d, 12.2 Hz, H-6), 4.25 (dd, 12.2, 4.28 Hz, H-6). Galloyl group δ 9a: 6.96 (d, 2H), 9b 6.92 (d, 2H),), 9c 6.93 (9, 2H),), 9d 6.94 (9, 2H), 9e 7.31 (d, 2H),), 9e' 7.25 (d, 2H). 13C-YaMR spectrum (CD3OD), glucose ring: 93.88 (C-1), 72.48 (C-5), 74.46 (C-3), 70.13 (C-4), 74.46 (C-5), 63.83 (C -6). Galloyl group: 166.32 (C-7a), 119.74 (C-8a), 110.76 (C-9a), 146.60 (C-10a), 140.82 (C-11a), 167.12 (C-7b), 120.74 (C-8b), 110.6 (C-9b), 146.57 (C-10b), 140.4 (C-11b), 167.31 (C-7c), 120.42 (C-8c), 110.45 (C-9c), 146.42 (C-10c) , 140.3 (C-11c), 167.12 (C-7d), 120.3 (C-8d), 110.42 (C-9d), 146.42 (C-10d), 140.34 (C-11d), 167.24 (C-7e), 121.14 (C-8e), 117.62 (C-9e), 147.55 (C-10e), 140.39 (C-11e). 166.72 (C-7e'), 120.56 (C-8e'), 110.96 (C-9e'), 146.66 (C-10e'), 140.56 (C-11e').

For hydrolysis products, 5% HCl was found to produce glucose and gallic acid, and comparing these results with literature data identified the substance as 6-O-bisgalloyl-1,2,3,4-tetra-O-galloyl-β-D-glucose (Taiwo et al., 2020). Additionally, hydrolysis in the presence of 5% HCl resulted in glucose and gallic acid in a 1:7 ratio, which was identified as 3,6-O-bisgalloyl-1,2,4-tri-O-galloyl-β-D-glucose (Hwang et al., 2000).

ANK-2 is amorphous, pale yellow powder, Rf 0.36 (system 1), liquid. h. 304-307 0C (with decomposition). pale yellow amorphous powder, (1-system), liquid (with decomposition). UB spectrum (MeOH, λmax, nm): 230 and 279. Mass spectrum ESI-MS negative analysis, m/z 1243 [M-H]-, MS/MS-decomposition products 1091, 939, 787, 635, 483, 331 , 169. 1H PMR spectrum (CD3OD), glucose ring: δ 6.31 (d, 8.3 Hz, H-1), 5.64 (d, 8.3 Hz, H-2), 6.06 (m, 9.6 Hz, H-3) , 5.72 (m, 9.6 Hz, H-4), 5.58 (dd, 9.6, 8.3 Hz, H2), 4.66 (m, H-5), 4.54 m (d, 12.2 Hz, H-6). Galloyl group δ 9a: 7.08 (d, 2H), 9b 7.01 (d, 2H),), 9c 7.04 (9, 2H), 9c' 7.20 (d, 2H), 9d 7.06 (9, 2H), 9e 7.21 (d, 2H),), 9e' 7.26 (d, 2H).13C-YaMR spectrum (CD3OD), glucose ring: 93.32 (C-1), 71.78 (C-2), 73.88 (C-3), 69.31 (C-4), 73.92 (C-5), 63.13 (C-6). Galloyl group: 164.92 (C-7a), 119.84 (C-8a), 110.16 (C-9a), 146.15 (C-10a), 140.1 (C-11a), 165.01 (C-7b), 119.84 (C-8b), 110.05 (C-9b), 146.02 (C-10b), 139.65 (C-11b), 165.9 (C-7c), 120.68 (C-8c), 1019.45 (C-9c), 144.42 (C-10c) , 139.3 (C-11c), 165.18 (C-7c'), 120.20 (C-8c'), 110.46 (C-9c'), 146.06 (C-10c'), 139.62 (C-11c'), 165.66 (C-7d), 119.79 (C-8d), 110.22 (C-9d), 146.1 (C-10d), 139.64 (C-11d), 165.82 (C-7e), 120.14 (C-8e), 117.36 (C -9e), 144.65 (C-10e), 139.78 (C-11e). 165.32 (C-7e'), 120.4 (C-8e'), 110.49 (C-9e'), 146.14 (C-10e'), 139.69 (C-11e').

It was found that in the products of hydrolysis carried out in the presence of 5% HCl, glucose and gallic acid are formed in a ratio of 1:7. Comparing the results obtained with literature data, this substance was identified as 3,6-O-bisgalloyl-1,2,4-tri-O-galloyl-β-D-glucose (Taiwo et al., 2020, Hwang et al., 2000)

The results of the mass, NMR, and HMWS spectra, as well as the biological and pharmacological activity of these isolated compounds, are currently being studied.

Statistical data processing and illustration design were carried out using the Origin 6.1 computer program (Microsoft, USA).

3. Results and Discussions

Dexamethasone-induced diabetes in aged rats allows for the recreation of fundamental pathophysiological mechanisms, including impaired insulin secretion and action, observed in type II diabetes mellitus (Ametov & Solov'eva, 2007; Atamanov, Yakovleva, & Tereshchenko, 2003; Balabolkin, Klebanov, & Kreminskaya, 2000). Hypoglycemic activity was assessed in 20 18-month-old white outbred rats, each with an initial body weight of 300-350 g, maintained on a high-fat diet. Prior to the experiment, the rats were weighed, and initial blood glucose levels were measured. Blood samples were collected from the tail to avoid excessive agitation. The rats were then divided into two groups of 10. Acute hyperglycemia was induced by a single intragastric administration of a hypertonic glucose solution at a dose of 5000 mg/kg. Blood glucose levels were measured 1 hour post-induction using the glucose oxidase method with a Cypress Diagnostic test kit (Germany) (Khoshimov et al., 2021).

In the experimental animals, blood glucose levels increased by 77%. The prothrombin time (PT), indicating the extrinsic pathway of coagulation, decreased by 100%, while the activated partial thromboplastin time (APTT), reflecting the intrinsic pathway, decreased by 46%. Fibrinogen levels increased by 41.4%, and clot weight increased by 50.0% compared to control values (Bondar, Klymentov, & Porshennikov, 2000; Kirichuk, Rebrov, & Kosheleva, 2002).

Three days after administering dexamethasone twice subcutaneously, biochemical parameters in the rats confirmed type 2 diabetes, with glucose levels increasing 2.65-fold ($p \leq 0.01$) compared to intact rats (3.1 ± 0.12 mmol/L). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels also increased by 3.2-fold ($p \leq 0.01$) and 2.2-fold ($p \leq 0.01$) compared to intact levels (31.5 ± 4.2 U/L and 49.2 ± 2.1 U/L, respectively) (Lyutova, Alekseeva, & Karabasova, 2002).

The effect of polyphenols ANK-1, ANK-2, and ITL-2 extracts on biochemical parameters modeled to type 2 diabetes was studied. Following five oral administrations of ITL-2 extract, all biochemical parameters normalized. Conversely, polyphenols ANK-1 and ANK-2 returned CV, ALT, glucose, total cholesterol, and triglyceride levels to intact values, but AST and ALT levels remained elevated (Stratman & Tschoehe, 2005; Taiwo et al., 2020).

A study on the impact of dexamethasone on hemostasis revealed that two subcutaneous doses led to significant reductions in PT, APTT, and plasma recalcification (RP) times, with increased

fibrinogen content compared to intact animals (Hunter & Hers, 2009; Sovalkin & Paymanov, 2005). Specifically, PT decreased by 32% (p = 0.00017), APTT by 33% (p = 0.008376), and RP by 32% (p = 0.030849), while fibrinogen content increased by 67.7% (p = 0.001262). These findings suggest that dexamethasone induces hypercoagulation in type II diabetes rats, affecting both the extrinsic and intrinsic pathways of blood coagulation and significantly increasing fibrinogen levels (Brummel, Jenny, & Mann, 2002; Nacag-Icindic, Valjevac, & Lepara, 2007; Raimova et al., 2021).

The antithrombotic effects of the studied drugs were evaluated by measuring the degradation of 50 µl of coagulated rat blood. The results, summarized in Table 3, show the absorption spectrum of the supernatant (blood clot plus saline solution).

In the control group, the coloration decreased by 28% (*p = 0.02610) compared to the intact group (0.640 ± 0.05). In contrast, the experimental groups exhibited different effects: with drug ANK-1, the coloration increased by 31.3% (**p = 0.01349); with drug ANK-2, by 21.1% (**p = 0.04347); and with drug ITL-2, by 37.5% (**p = 0.001547) relative to the control (0.460 ± 0.04).

Regarding thrombus weight, the control group exhibited an increase of 11% (*p = 0.29552 mg) compared to the intact group (16.2 ± 1.0 mg). Treatment with ANK-1 resulted in a 22.5% (**p = 0.02742) reduction in thrombus weight, ANK-2 showed a minimal reduction of 4.4% (**p = 0.64061), and ITL-2 led to a significant reduction of 39.7% (**p = 0.000001) compared to the control group (18.2 ± 1.3 mg).

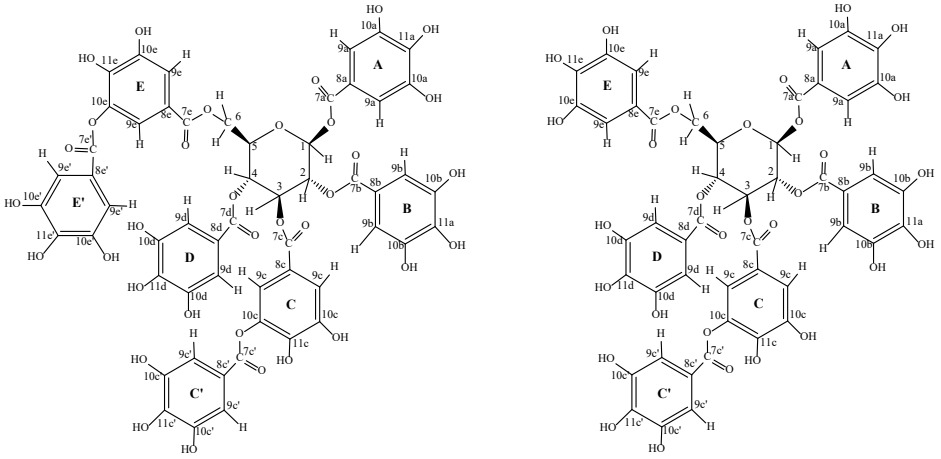
These findings indicate that hypercoagulation is present in rats with type II diabetes mellitus, manifesting as a 30-40% increase in external prothrombin time (PT) and internal activated partial thromboplastin time (APTT), and a 60-70% increase in fibrinogen content. Oral administration of the studied drugs at doses of 10-15 mg/kg normalized the blood clotting process after three doses. Among the drugs, ANK-1 and ITL-2 exhibited the most pronounced antithrombotic effects, with activity increases of 31.3% and 37.5%, respectively, and clot inhibition rates of 22.5% and 39.7% following three administrations.

As is well-established, thrombosis pathogenesis involves mechanisms interrelated with impaired carbohydrate metabolism, including hyperglycemia, insulin deficiency, and insulin resistance, which contribute to metabolic and cellular disorders (Nacag-Icindic, Valjevac, & Lepara, 2007). Chronic hyperglycemia plays a critical role in platelet dysfunction. Elevated blood glucose levels lead to the glycation of platelet surface proteins, resulting in structural changes in platelets and increased adhesive properties (Winocour, Watala, Perry, & Kinlough-Rathbone, 1992). These effects are mediated through various mechanisms. One such mechanism involves the substrate of the insulin receptor (SIR), a large cytoplasmic protein central to many receptor systems, including insulin-like growth factor-1 (IGF-1) (Hunter & Hers, 2009). Platelets express both SIR and IGF-1, stimulating phosphorylation of these receptors and protein kinase C in a dose-dependent manner. Increased insulin resistance amplifies the effect of SIR and IGF-1 on platelet aggregation (Ishida et al., 1996). Another insulin-mediated mechanism is the rise in intracellular calcium concentration and its ionization, which is significant in insulin resistance and relative insulin deficiency (Martyanov et al., 2020). Insulin resistance reduces the expression of the prostacyclin receptor on platelets, enhancing their aggregation properties (Brummel, Jenny, & Mann, 2002).

Diabetes is characterized by increased platelet activity driven by hyperglycemia, endothelial dysfunction, insulin deficiency or resistance, dyslipidemia, oxidative stress, and inflammation (Carr, 2001). Consequently, chronic hyperglycemia notably disrupts platelet hemostasis. In this context, the next stage of our research involved studying the effects of polyphenols ANK-1, ANK-2, and ITL-2 on platelet function in rats with experimentally induced type 2 diabetes mellitus. Previous in vitro studies showed that spontaneous platelet aggregation was not observed in these rats, which is consistent with findings that spontaneous aggregation remains within the normal range or increases slightly in type 2 diabetes (Ametov & Solov'eva, 2007). However, platelet aggregation in response to ADP, adrenaline, collagen, and ristocetin is typically increased in this condition (Atamanov, Yakovleva, & Tereshchenko, 2003; Balabolkin, Klebanov, & Kreminskaya, 2000). When ADP, adrenaline, collagen, and ristocetin inducers were added to the plasma of rats with type 2 diabetes, platelet aggregation was observed to vary with the concentration (5-10 mmol). Notably, the most significant aggregation was seen with ADP and epinephrine agonists (see Figure 2). At higher concentrations (10 mmol), ADP inducers caused irreversible platelet aggregation, presenting as a two-phase curve with primary and secondary aggregation (Figure 3a). Similarly, adrenaline inducers (1-5 mmol) dose-dependently induced platelet aggregation. At a concentration of 2 µmol, adrenaline caused a two-phase curve, while at higher concentrations, it led to irreversible platelet aggregation in rats with type 2 diabetes (Figure 3b).

When examining the effects of polyphenols ANK-1 (50 µg/ml), ANK-2 (50 µg/ml), and ITL-2 (50 µg/ml) on platelet aggregation induced by ADP, a significant inhibitory effect was observed with ANK-1 and ANK-2 (Figure 4). At lower concentrations (up to 10 µg/ml), ANK-1 and ANK-2 did not significantly inhibit ADP-induced primary and secondary platelet aggregation. However, at concentrations above 10 µg/ml, they inhibited platelet aggregation by over 50%, reducing both the maximum aggregation values and the rate of aggregation, as indicated by the maximum slope of the curve (Figure 5).

ADP binds to its receptors P2Y1 and P2Y12 on platelet membranes, leading to changes in platelet shape and initiating aggregation. This



A B

Figure 1. Pistacia vera plant phenolic compounds. A) 3,6-bis-galloil-1,2,4-to'rtgalloil-β-D-glyukoza, B) 3-bis-galloil-1,2,4,6-to'rtgalloil-*β-D*-glyukoza.

Table 1. Glucose parameters, coagulograms and clot weight in rats (M±m, n=5)

Tests	**CONTROL**	**Nutritional hypoglycemia**	**P**
Glucose	3,1±0,12	9,2±0,5	p=0.000009
Prothrombin time, (PT), sec	22,1±2,0	11,0±1,0	p=0.000118
Activated partial thromboplastin time (aPTT), sec	44,2±3,0	30,3±2,1	p=0.001444
Plasma recalcification, sec	36,4±2,5	24,6±2,2	p=0.002491
Fibrinogen, g/dl	315,6±13	446,2±25	p=0.000237
Plasma extinction 410 n	0,64±0,05	0,56±0,04	p=0.228452
Weight of clots. mg	6,8±0,5	10,2 ±1,0	p=0.007379

** P≤0.05 relative to the control group of animals.

Table 2. Effect of drugs ANK-1, ANK-2 and ITL-2 on biochemical blood parameters in rats after subcutaneous administration of dexamethasone (M±m, n=5)

Research groups	УО, g/ l	АЛТ, U/ l	АСТ, U/ l	Glucose, mmol/l	General cholesterol, mmol/l	Triglycerides mmol/l
Intact	25,4±2,8	31,5±4,2	49,2±2,1	3,1±0,12	*1,8±0,08*	*0,87±0,02*
Indicators on the 3rd day from the beginning	24±1,3 *p=0.661	101±7,9 *p=0.0001	107±14 *p=0.0027	9,2±0,5 *p=0.00012	4,1±0,12 *p=0.0341	2,03±0,1 *p=0.1511
5 times oral administration of polyphenols and extract						
	УО, г/л	АЛТ, U/л	АСТ, U/л	Glucose, mmol/l	General cholesterol, mmol/l	Triglycerides mmol/l
Intact	25,4±2,8	31,5±4,2	49,2±2,1	3,1±0,12	*1,8±0,08*	*0,87±0,02*
after administration of dexamethasone	24,4±2,0	111,6±6,2*	109,2±9,1 *	9,2±0,5 *	4,1±0,12 *	2,03±0,1 *
ANK-1	25,0±2,1	41,5±4,2 **	58,2±2,8 **	3,4±0,41 **	*1,9±0,11*	*0,89±0,07*
ANK-2	24,8±2,2	33,5±3,2 **	50,2±2,4**	3,6±0,41**	*1,79±0,12*	*0,86±0,06*
ITL-2	24,6±2,6	31,8±3,5 **	49,0±2,1**	3,0±0,41**	*1,82±0,14*	*0,87±0,05*

* p ≤0.05 in relation to the intact group of animals; **p ≤0.01 relative to the control group of animals.

Table 3. The effect of ANK-1, ANK-2 and ITL-2 drugs on coagulogram parameters in rats after subcutaneous administration of dexamethasone (M = m, n=5)

Drugs	Coagulogram indicators			
	Prothrombin time, (PT), sec	Activated partial hrombotic plate time aPTT), sec	Plasma recalcification, sec	Fibrinogen, g/dl
intact	22,0±1,4	40,6±3,0	46,4±2,5	325,8±24
Outcome of diabetes	12,9±1,15 *p=0.001526	21,7±1,9 *p=0.001096	30,3±2,3 *p=0.115614	625,0±35 *p=0.000202
5 times oral administration of polyphenols and extract				
CONTROL	12,9±1,15 *p=0.001526	21,7±1,9 *p=0.001096	30,3±2,3 *p=0.115614	625,0±35 *p=0.000202
ANK-1	19,6±1,5 *p=0.28040 **p=0.009409	39,9±2,8 *p=0.86938 **p=0.001032	44,4±9,5 *p=0.000300 **p=0.000166	367,8±24 *p=0.255821 **p=0.000511
ANK-2	28,0±1,7 *p=0.02957 **p=0.000155	26,3±2,0 *p=0.005419 **p=0.139351	36,0±2,6 *p=0.119274 **p=0.933551	371,5±29 *p=0.264099 **p=0.000836
ITL-2	23,7±1,5 *p=0.43470 **p=0.00117	27,9±2,1 *p=0.003672 **p=0.059417	42,9±3,3 *p=0.160399 **p=0.016553	339,3±24 *p=0.702667 **p=0.000269

**p ≤0.05 in relation to the intact group of animals; **p ≤0.01 in relation to the control group of animals.*

Table 4. Antithrombotic activity after administration of ANK-1, ANK-2 and ITL-2 in rats with dexamethasone diabetes (M±m, n=5)

TEST	**INFACT**	**CONTROL**	**ANK-1** **10 mg/kg**	**ANK-2** **10 mg/kg**	**ITL-2** **15 mg/kg**
Plasma extinction 410 nm	0,640±0,05	0,460±0,04	Plasma extinction 410 nm	0,640±0,05	0,460±0,04
Antithrombotic activity, %		*-28	+31,3**	+21,1**	37,5**
Weight of clots. mg	16,2±1,0	18,2±1,3 *p=0.29552	14,1±0,7 *p=0.15129 **p=0.0274	17,4±1,0 *p=0.46747 **p=0.6406	12,8±0,6 *p=0.03004 **p=0.00696
Clot induction or inhibition, %		*+11	**-22,5	**-4,4	**-39,7

*Note: *p <in relation to the intact; **p< in relation to the control group.*

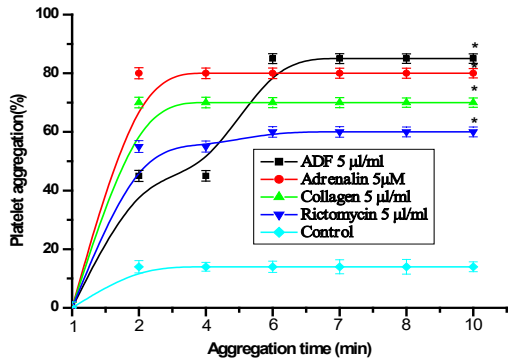


Figure 2. Effect of platelet aggregation inducers on the blood plasma of rats experimentally modeled with type 2 diabetes.*- P <0.05(n=6)

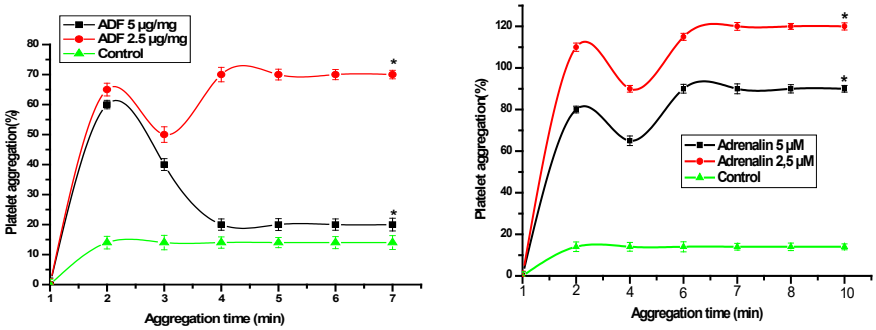


Figure 3. Effect of platelet aggregation inducers ADP and adrenaline on the blood plasma of rats experimentally modeled with type 2 diabetes mellitus**.** *- P <0.05; (n=6).

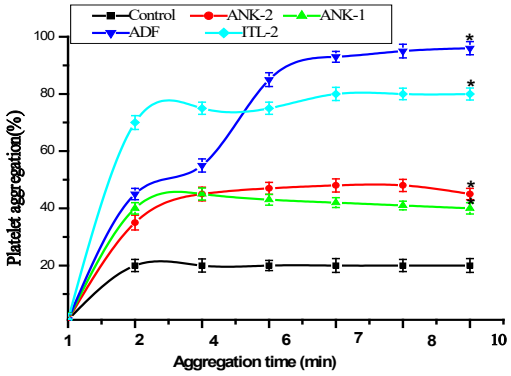


Figure 4. Effect of polyphenols ANK-1, ANK-2 and ITL-2 on ADP-induced platelet aggregation. *- P <0.05; (n=6).

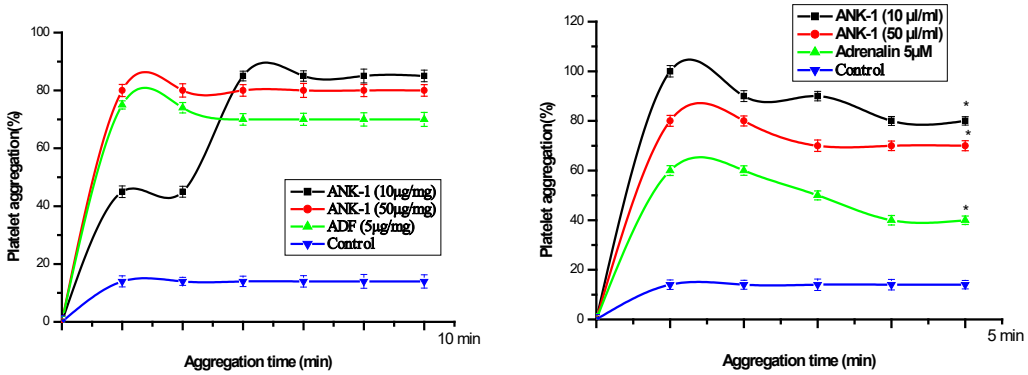


Figure 5. Effect of polyphenols ANK-1, ANK-2 on ADP and adrenaline-induced platelet aggregation depending on dose. *- P<0.05. (n=6).

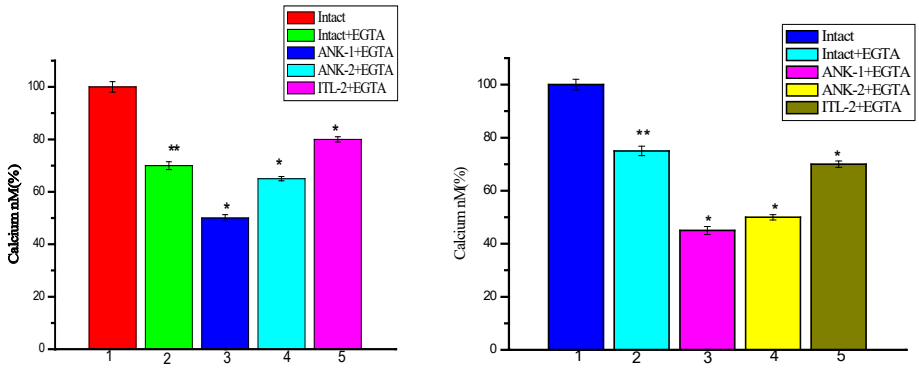


Figure 6. Effect of the compound ANK-1, ANK-2 and EGTA on the binding of calcium ions in intact platelets and experimentally modeled type 2 diabetes mellitus. Column 1 – control (platelets were incubated with 5 μM Fura–2/AM for 30 min at a temperature of 37°C in the absence of Ca2+ ions). 2–3 columns – platelets were incubated with 5 μM Fura–2/AM for 30 min at a temperature of 37°C in the absence of Ca2+ ions against the background of EGTA and EZK, respectively. Reliability indicator * – p<0.05; **– p<0.01; (n=6).

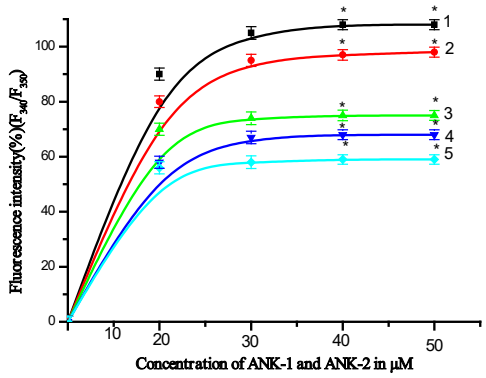


Figure 7. Effect of polyphenols ANK-1, ANK-2 on the background of verapamil when indicating changes in cytosolic calcium in platelets with ADP and adrenaline. 1- ADP fluorescence, 2- adrenaline fluorescence, 3-verapamil on ADP induced, 4-ANK-1 on ADP, 5-ANK-2 on ADP. *- P<0.05. (n=6).

process is likely due to the inhibition of adenylate cyclase, which reduces cAMP production and increases intracellular calcium ion ($[Ca^{2+}]$) release from storage depots, resulting in elevated cytoplasmic calcium levels (Ishida et al., 1996). The third stage of this response includes visible platelet aggregation and the release of various chemicals from the cells. Adrenaline, by interacting with its receptors, also significantly inhibits adenylate cyclase, enhancing cell membrane permeability and calcium ion transport (Stratman & Tschoehe, 2005). Consequently, increased cytosolic calcium activates protein kinases and leads to the phosphorylation of regulatory proteins within platelets. A sharp rise in cytosolic calcium from the endoplasmic reticulum is crucial for platelet activation, including aggregation, secretion, and the release of coagulation factors. The mobilization of intracellular calcium ions is a key element in this process, though different initiators of aggregation may induce this mechanism differently (Randriamboavonjy & Fleming, 2009).

Recent studies have shown that insulin resistance (IR) underlies many receptor systems, including those similar in structure to proinsulin, such as insulin-like growth factor-1 (IGF-1). Platelets express both IR and IGF-1, which stimulate the phosphorylation of IGF-1 receptors, tyrosine residues of IR, and protein kinase C (Hunter & Hers, 2009). This effect is dose-dependent: greater insulin resistance enhances the impact of IR and IGF-1 on platelet aggregation (Nasirov et al., 2023). Additionally, insulin-mediated mechanisms in diabetes lead to increased intracellular calcium levels and ionization. Normally, ionized calcium activates enzymes such as phospholipase C and A, initiating the arachidonic acid cascade and the formation of thromboxane and prostacyclin (Hwang et al., 2000). However, in insulin resistance and relative insulin deficiency, interactions with platelets through IR and IGF-1 reduce prostacyclin receptor expression, disrupting the balance between the aggregation inhibitor prostacyclin and the aggregator thromboxane (Carr, 2001).

Given this context, polyphenols ANK-1 and ANK-2 likely act by activating adenylate cyclase, decreasing thromboxane formation, and influencing calcium ion mobilization from intracellular depots. These polyphenols more effectively inhibit the second wave of ADP and adrenaline-induced platelet aggregation, likely due to their impact on thromboxane activation and intracellular calcium concentration (Raimova, Khoshimov, Nasirov, & Turaev, 2021).

To investigate whether the antiplatelet effects of ANK-1 and ANK-2 polyphenols are associated with the inhibition of calcium ion (Ca^{2+}) entry and mobilization from intracellular stores, we assessed their impact on membrane-bound and cytosolic calcium levels using fluorescence probes, chlortetracycline (CHTC) and Fura-2. The experiments were conducted in two stages, examining the effects both in the presence and absence of physiological calcium concentrations.

Initially, we studied the chelating ability of ANK-1 and ANK-2 polyphenols concerning calcium ions in rat platelets, with and without EGTA, under stimulation by ADP and adrenaline. Our results indicated that ANK-1 and ANK-2 polyphenols effectively block the mobilization of intracellular calcium into the platelet cytoplasm (Figure 6).

Subsequently, when platelets were stimulated with ADP and adrenaline, the release of Ca^{2+} from cellular stores was dependent on the concentration of the inducers, which served as the control. We observed a reduction in cytosolic calcium levels when using the calcium channel blocker verapamil, which inhibits inositol trisphosphate (IP_3)-controlled calcium channels. In the presence of ANK-1 and ANK-2 polyphenols (10-50 μg/ml) added two minutes before ADP induction, there was a consistent decrease in the mobilization of calcium ions from intracellular depots, irrespective of the dose (Figure 7). Notably, in the presence of verapamil, ANK-1 and ANK-2 polyphenols (50 μg/ml) also reduced calcium mobilization from intracellular stores.

These findings suggest that ANK-1 and ANK-2 polyphenols do not primarily act on IP_3-controlled platelet calcium channels. The release of calcium is regulated by several factors. When agonists bind to their respective receptors on the platelet membrane, intermediates are generated that stimulate calcium release from storage sites. Thromboxane A_2 (THAg) further promotes calcium release by inhibiting adenylate cyclase activity, which results in decreased cyclic AMP (cAMP) levels. Under normal conditions, cAMP inhibits calcium release from the endoplasmic reticulum (ER); therefore, a decrease in this inhibition due to THAg action leads to an increased release of calcium into the cytoplasm.

Conclusion

In conclusion, this study demonstrates that dexamethasone-induced type 2 diabetes in rats significantly disrupts coagulation pathways, leading to hypercoagulation, as evidenced by increased fibrinogen levels and altered coagulation times. The administration of polyphenolic compounds, including Hexagalloyl-glucose (ANK-1), Heptagalloyl-glucose (ANK-2), and Isatis tinctoria L. (ITL-2), revealed their potential in modulating these effects. Notably, ANK-1 and ITL-2 showed pronounced antithrombotic effects, effectively reducing thrombus weight and clot density compared to the control. These compounds also demonstrated significant inhibition of platelet aggregation induced by ADP and adrenaline, likely due to their impact on intracellular calcium mobilization and thromboxane formation. These findings underscore the therapeutic potential of polyphenolic compounds in managing diabetes-induced hypercoagulation and warrant further exploration to validate their efficacy and safety in clinical settings.

Author contributions

R.G.M. conceptualized and supervised the study. N.K.E. and K.S.S. contributed to data analysis and interpretation. O.M.M. and M.R.R. assisted in drafting sections of the manuscript. T.M.A. and I.S.M. were involved in the research design and provided critical revisions. U.M.S. supported data collection and manuscript finalization. All authors reviewed and approved the final version of the manuscript.

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Competing financial interests

The authors have no conflict of interest.

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