Chapter

Galega officinalis L. and Immunological Status in Diabetes Mellitus

Mariia Nagalievska, Halyna Hachkova and Nataliia Sybirna

Abstract

Under diabetes mellitus, the administration of *Galega officinalis* promotes restoration of leukocyte precursors' bone marrow pool and normalizes their proliferative activity. This plant protects the functional state of leukocytes by modulating actin cytoskeleton formation and through quantitative redistribution of leukocyte membrane glycoconjugates. *Galega officinalis* prevents the development of diabetes-associated oxidative stress which results in antiapoptotic activity. The normalization of leukocytes' proliferative and functional capacity by *Galega officinalis*, along with its antiapoptotic and hypoglycemic effects, can improve the course of the disease and may prevent the development of complications of diabetes.

Keywords: *Galega officinalis*, diabetes mellitus, leukocytes, immune system

1. Introduction

Diabetes mellitus belongs to a group of metabolic diseases accompanied by chronic inflammation and attenuation of the immune response, which subsequently contributes to the development of a number of complications [1]. Cells that are most affected by glycemic status and insulin level are leukocytes, which play major roles in inflammation and immune responses [2]. Constant high glucose levels result in the formation of cytotoxic compounds, leading to lower viability of peripheral blood leukocytes. This is mediated by enhanced reactive species production, activation of mitogen-activated protein kinase (MAPK) pathway, high levels of pro-inflammatory and poly (adenosine diphosphate [ADP]-ribose) polymerase (PARP) transcription factors, as well as inactivation of pro-survival pathways which altogether leads to increased apoptosis rate. The alterations in these molecular pathways are usually associated with increased leukocyte mobilization, which causes changes in their morphology and functional state [1, 3].

The multitude of diabetes mellitus complications creates the need for drugs with a wide spectrum of action, which would not only provide effective reduction of blood glucose but would also exhibit cytoprotective properties. The most commonly used anti-diabetes drug globally is metformin. Metformin shows a pleiotropic effect mediated by its hypoglycemic function, as well as inhibitory effect on oxidative stress and inflammation.

In many cases medicinal plants can be safe and effective alternatives to synthetic compounds in disease management, since they possess a unique composition of biologically active substances [4].

Galega officinalis (*Galega*, goat's rue, French lilac) is a promising plant that can be used for treatment of a wide range of inflammatory diseases, including diabetes mellitus. *G. officinalis* is well-known for its hypoglycemic action, and it has been long used as part of a plant mixture for treatment of diabetes mellitus [5]. For a long time, the antidiabetic effect of *G. officinalis* was associated with high content of alkaloid galegine, which is one of the main components of this plant's leaves. In fact, metformin, discussed above, is a synthetic form of galegine, which was originally used to treat diabetes mellitus type 2 [5]. The toxicity of *G. officinalis'* alkaloids decreased its attractiveness as a hypoglycemic drug. However, it was found that even the non-alkaloid extract has a hypoglycemic effect and is potentially nontoxic [6, 7]. Based on such historical use and a large number of recent scientific studies, *G. officinalis* is a source of potent biologically active substances for the prevention and treatment of diabetes mellitus [8].

2. Effects of metformin on the immune system

Metformin (N,N-dimethylbiguanide) is an oral antihyperglycemic agent, which from a chemical point of view is a synthetic derivative of guanidine. The hypoglycemic effect of this drug is realized through the inhibition of hepatic glucose production, reducing intestinal glucose absorption and improving glucose uptake and utilization by peripheral tissues. Recent research has shed light on the pleiotropic effect of metformin, ranging from hypoglycemic function to cardio- and nephroprotection, as well as inhibitory effects on oxidative stress and inflammation [9–11].

The scientific data concerning the influence of metformin on the immune system is controversial, and its effect strongly depends on the pathology in which it is used. For example, metformin enhances antitumor immunity, but in other contexts, it can act as an anti-inflammatory or immunosuppressive agent [8]. Metformin can suppress senescence- and cancer-related inflammation. The majority of experimental data indicates that metformin modulates leukocytes' functional activity by activating 5′ adenosine monophosphate-activated protein kinase (AMPK). Metformin can activate AMPK in multiple cell populations, including macrophages and neutrophils [12, 13]. It has also been demonstrated that metformin inhibits innate immune response to fungal infection in an AMPK-dependent manner and lessens central nervous system inflammation [14].

Considering the significant modulating effect of metformin on the immune system, it is unsurprising that it has a strong effect on immunocompetent blood cells, which we discuss below.

2.1 Metformin influence on defective hematopoiesis

Studies conducted on Fanconi anemia mice showed the unique property of metformin to improve hematopoiesis by restoring hematopoietic stem cell (HSC) numbers. It also delays tumor formation, presumably via reduction of DNA damage induced by aldehydes [15]. An important part of metformin protective effect may be conferred by aldehyde detoxification. Other mechanisms by which metformin may act to protect the cell's DNA are reducing the activity of mitochondrial complex 1 activity, thus potentially reducing oxidative DNA damage. It is also possible that metformin can switch the metabolic balance between oxidative phosphorylation and anaerobic glycolysis and downregulate inflammatory pathways which are thought to contribute to bone marrow failure [15]. Another study demonstrates that metformin treatment significantly inhibited the total-body irradiation-induced increase in the levels of DNA double-strand breaks and reactive oxygen species

(ROS) by attenuation of NOX4 expression in HSCs. Furthermore, metformin modulates the expression of antioxidant enzymes in HSCs [16].

2.2 Influence of metformin on functional state of leukocytes

Many diabetic patients who receive metformin show significantly reduced neutrophil-to-lymphocyte ratio [9]. Metformin is able to reduce hyperneutrophilia in girls with hyperinsulinemic hyperandrogenism and improves white blood cell count in women with polycystic ovary syndrome, two conditions characterized by a pronounced systemic inflammatory state [17]. Metformin increased the number of CD8-positive tumor-infiltrating lymphocytes. Normalizing effect of metformin on the number of immunocompetent cells is associated with its ability to upregulate AMPK and as a consequence of altering energy metabolism in the cell [14].

Apart from metformin influence on immunocompetent cell number, this drug also can modulate their functional activity. As expected for an AMPK activator, metformin enhances cell mobility and phagocytosis, in particular in macrophages that show enhanced uptake of bacteria, synthetic beads, or apoptotic cells. The effects of AMPK activation may be due to its ability to increase availability of cell surface receptors, including αM integrin or Fc receptors or due to mechanisms that involve suppression of TLR4-associated signaling pathways. Metformin by activating AMPK regulates the process of inflammation resolution—efferocytosis and enhanced uptake of bacteria by phagocytic cells [12, 13].

Additionally, in patients with prediabetes, metformin treatment reduces the concentration of neutrophil extracellular trap (NET) components independently from glycemic control [14].

The normalization of phagocytosis processes and NETosis under metformin administration could suggest an effect of this drug on neutrophil activation. Indeed, metformin attenuates neutrophil activation via inhibition of mitochondrial respiratory complex I, potentially through intracellular H_2O_2-mediated inhibition of IκB-α degradation and thus prevention of NF-κB activation [18].

Immune system modulation by metformin can be realized not only by its direct influence on the immunocompetent cells but also by its ability to regulate chemokine level. Metformin causes a decrease in inflammatory markers in plasma, including soluble intercellular adhesion molecule, vascular cell adhesion molecule-1, macrophage migration inhibitory factor, C-reactive protein, IL-6, and IL-8. The anti-inflammatory action of metformin is realized by suppressing Akt, Erk1/2, and NF-B translocation. Such changes lead to blocking of pro-inflammatory signal transduction via the phosphoinositide 3 kinase pathway [19].

Immunosuppressive effect of metformin can be mediated by its ability to inhibit the expression of pro-inflammatory mediators (IFN-, TNF-, IL-1, IL-6, IL-17, iNOS, MMP9, and RANTES) and infiltration of immune cells, which was blocked by reducing the expression of CAMs (ICAM, VCAM, and E-selectin) on vascular cells [20, 21].

2.3 Effects of metformin on oxidative stress

Oxidative stress is the leading cause of microvascular and cardiovascular diabetes complications [22]. Disruption of glucose metabolism causes mitochondrial superoxide overproduction in cells. An increased amount of superoxide leads to overactivity of polyol and hexosamine pathways, increased formation of AGEs (advanced glycation end products) and its receptors, and activation of protein kinase C isoforms. Altogether, this leads to the development of complications of diabetes. Simultaneously endothelial nitric oxide synthase is inactivated. Changes

in the activity of these signaling pathways result in increased intracellular ROS and activation of pro-inflammatory pathways [22].

Considering such intimate link between diabetes and oxidative stress, anti-diabetes treatments should not only reduce blood sugar but should also possess strong antioxidant properties. Metformin satisfies both criteria; as in addition to a hypoglycemic effect, it improves the immunological parameters of patients, presumably through its antioxidant properties [23]. In aortic endothelial cells, metformin has been shown to inhibit high glucose-dependent ROS overproduction, which was mediated by a reduction in NADPH oxidase activity and an inhibition of the respiratory chain complex 1. Another possible mechanism of metformin antioxidant properties is its ability to activate AMPK with the ensuing induction of manganese superoxide dismutase and expression of the antioxidant thioredoxin and endothelial NO synthase (eNOS). Additionally, metformin is able to reduce AGEs synthesis and the expression of their specific cell receptor called RAGE in endothelial cells [16, 23]. In addition to the abovementioned indirect mechanisms of modulation of superoxide anion intracellular production, it was found that metformin can directly scavenge ROS, in particular $^{\bullet}OH$ but not $O_2^{\bullet}$ [16].

While leukocytes actively participate in ROS generation, they are highly sensitive to ROS-mediated oxidative damage. Metformin was demonstrated to have a protective effect against oxidative stress in immunocompetent cells [24].

Furthermore, metformin modulates the function of fMLP-activated polymorphonuclear neutrophils that quench the products of oxidative burst. Researchers hypothesized that metformin may recognize specific cell membrane sites, thereby inducing intracellular signal transduction resulting in changes in NADPH oxidase activity or in other sources of intracellular ROS [25]. Furthermore, metformin-induced decrease in ROS levels led to a partial inhibition of lipid peroxidation in lymphocytes [26].

2.4 A protective role of metformin against apoptosis

Most chronic diseases, including diabetes mellitus, are accompanied by oxidative stress, which may result in apoptosis of different types of cells [27]. Metformin has been shown to have protective role on apoptosis. The inhibition of apoptosis by metformin has been described in many cell types and under various conditions. There may be several mechanisms of apoptosis prevention. Firstly, metformin possesses good radical scavenging activity. Secondly, metformin can regulate caspase levels and induce xenobiotic phase II enzymes [28].

A number of authors have concluded that metformin exerts a neuroprotective effect by decreasing mitochondria-dependent apoptosis. This is achieved through the inhibition of permeability transition pore opening, blocking the release of cytochrome c and preventing subsequent cell death [29]. A protective role of metformin against programmed cell death is likely mediated by maintaining mitochondria integrity and reducing Ca^{2+}. This drug also lowers the expression of caspase-3, cytochrome c, and cleaved caspase-9 and reduces fragmentation of PARP-1 while increasing the expression of Bcl-2 [29]. A similar protective effect of metformin has been described for primary rat hepatocytes. Metformin may protect against apoptosis by induction of menadione-induced heme oxygenase-1 and bcl-xl expression and the reduction of c-Jun N-terminal kinase activation [30, 31].

Given the ability of metformin to inhibit apoptosis of different cells in a variety of pathologies, it is possible to assume that it has a similar effect on immunocompetent blood cells. Indeed, it was shown that metformin markedly decreased the percentage of apoptotic cells in bone marrow cells of rats [32]. It also reduces the activation of macrophages and inhibits the expression of COX-2 and caspase-3, thereby attenuating inflammatory responses and apoptosis [33].

Treatment with metformin reduces the amount of oxidant-induced DNA damage in lymphocytes. It was shown that pharmacological concentration (50 μM) of metformin could protect against prooxidant stimulus-induced DNA damage at early but not late stages. Thus, metformin likely exerts an antiapoptotic effect by reducing caspase-3 and caspase-8 activities [28].

3. Effects of *Galega officinalis* L. on immunocompetent cells under diabetes mellitus

Galega officinalis (goat's rue) is a toxic leguminous plant originated in the Eastern Mediterranean and Black Sea regions but now has been spread in southeastern parts of Europe and the Middle East. In the medieval period, this plant was traditionally used for the treatment of diabetes [5, 34]. *G. officinalis* contains a large number of secondary metabolites with pronounced biological properties, among which are alkaloids, saponins, flavonoids, tannins, fatty acids, and phytoestrogens [35].

3.1 Component composition and hypoglycemic effect of non-alkaloid extract of *Galega officinalis*

The non-alkaloid extract of *G. officinalis* can be obtained by a two-step extraction [6, 7]. In the first stage, the biologically active substances are obtained by plant material infusion in 96 % ethanol. After alcohol evaporation, equal volumes of water and chloroform are added to the residue. The obtained chloroform fraction should be evaporated to obtain the solid residue, which is then dissolved in water to form an emulsion. The latter is not stable and eventually forms a precipitate. The stability of emulsions is very important; their stratification affects the accuracy of active substance content measurement. To solve this problem, the biocomplex PS (surface-active products of *Pseudomonas* sp. PS-17 biosynthesis) can be used [7]. Using gas chromatography/mass spectrometry method, it was established that the biocomplex PS consists of methyl ester of decenoic acid and dodecenoic acid. These surfactants were added to the initial mixture obtained by the addition of water to non-alkaloid fraction of *G. officinalis*. Such extraction and stabilization yield a stable water emulsion without toxic alkaloids [6, 36].

Crucially, such non-alkaloid fraction of *G. officinalis* extract exhibited a hypoglycemic effect in streptozotocin-induced diabetes mellitus if administered for 14 days at 600 mg/kg per day. Notably, blood glucose concentration decreased to physiological values [6, 7].

Blood glucose measurement evaluates current glucose concentration, which may depend on many factors (the intake and composition of food, physical activity and their intensity, the emotional state of the patient, and even the time of the day) [37]. Thus, blood glucose concentration may not reflect the actual degree of diabetes compensation, potentially resulting in medication under- or overdosing. Therefore, today, the key indicator for treatment quality and risk of diabetes complications is the level of glycosylated hemoglobin (HbA1c) [37]. Notably, the non-alkaloid fraction of *Galega officinalis* extract normalizes HbA1c content under diabetes [6].

Sugar-reducing effect of non-alkaloid extract may be due to its complex composition [6, 36, 38]. Gas chromatography/mass spectrometry detected phytol as a component of non-alkaloid fraction of *Galega officinalis* extract. Phytol might contribute to the extract's sugar-lowering effect, as it is known to lower insulin resistance and sensitivity of muscles to insulin and to reduce gluconeogenesis [39]. It has been shown that phytol can increase the expression of *GLUT2* and *glucokinase*

genes through activation of RXR (retinoid X receptor) [39], which are otherwise downregulated under diabetes mellitus. Palmitic acid esters in the extract could also cause a dose-dependent decrease in blood plasma glucose in animals with experimental diabetes mellitus [40]. Furthermore, non-alkaloid fraction of *Galega officinalis* extract contains high levels of phytosterols (campesterol and stigmasterol) that, in addition to the ability to inhibit cholesterol adsorption, can reduce the level of glycosylated hemoglobin [41, 42].

Another notable biologically active substance from *Galega officinalis* is α-amyrin. It has a hypoglycemic action and can influence endocannabinoid system. Some ligands for cannabinoid CB1 receptors can directly bind and allosterically regulate Kir6.2/SUR1 K (ATP) channels, thereby controlling glucose-stimulated insulin release. In addition, α- and β-amyrin, due to their anti-inflammatory and antioxidant properties, have a positive effect on the state of animals with streptozotocin diabetes [43].

It has been shown that quinazoline derivatives are capable to lower blood glucose level and body weight in obese animals [44]. Notably, the non-alkaloid fraction of *Galega officinalis* contains such substances (2-methyl-1,2,3a,4,5-hexahydropyrrolo[1,2-a]quinazoline). These derivatives can increase the activity of AMPK, which results in increased glucose adsorption by muscle cells. It has been found that AMPK, in addition to regulating insulin release by pancreatic cells, inhibits the activity of acetyl-CoA-carboxylase and hydroxymethylglutaryl-CoA-reductase in fat cells, thereby inhibiting the biosynthesis of fatty acids and cholesterol [45].

High content of alpha-linolenic acid in *Galega* extract is also noteworthy. Omega-3 polyunsaturated fatty acids increase cell membrane fluidity, as well as the number of insulin receptors, the affinity of insulin to these receptors, and the number of type 4 glucose transporters; they also regulate the balance between pro- and antioxidants [46].

Based on the above statement, the sugar-lowering effect of the non-alkaloid fraction of *Galega officinalis* extract is likely due to the presence of phytol, ethyl ester of palmitic acid, phytosterols (campesterol and stigmasterol), and quinazoline derivatives, acting separately or synergistically [6].

3.2 Regulation of bone marrow cells proliferation by *Galega officinalis*

Many of diabetes complications are induced by the intensification of chronic inflammation and attenuation of the immune response. Leukocytes play major roles in inflammation and immune responses. Diabetes mellitus is accompanied by infectious and inflammatory processes, of which the most frequent are bacterial infections, which are accompanied by relapses and are difficult to treat. Changes in the proliferative activity and ratio of leukocytes and changes in their functional properties and activation of free radical oxidation are among probable causes of the propensity of patients with diabetes mellitus to infectious processes and their compromised immunological status [2].

Therefore, the measurement of the hypoglycemic effect is insufficient when testing the effectiveness of new antidiabetic agents. It is also necessary to evaluate the effect of potential hypoglycemic drugs on cells that are susceptible to metabolic changes in diabetes mellitus. Cells whose function is very significantly affected in the course of diabetes mellitus are white blood cells. High levels of glucose in the bloodstream cause inflammation, which primarily affects blood cells, in particular, leukocytes [47, 48].

In addition to a broad spectrum of substances with a hypoglycemic effect, the non-alkaloid fraction of *Galega officinalis* extract contains compounds with potential immunomodulatory effect. *Galega officinalis* normalizes differential count of

leukocytes in conditions of diabetes mellitus. In particular, it leads to an increase in the number of segmented and band neutrophils while overall lowering the number of lymphocytes to almost control values [49]. This indicates a normalization of the cell-mediated immune response, as one of the most important factors determining the activity of the immune system of an organism [49]. The normalization of the content of immunocompetent cells in blood after treatment of diabetic rats with *Galega* extract may be due to the influence of its biologically active substances on the proliferation of these cells.

The non-alkaloid fraction of *Galega officinalis* extract, as a source of biologically active substances with wide range of actions, significantly affects the proliferative activity of bone marrow cells in conditions of diabetes. In particular, in rats with streptozotocin-induced diabetes mellitus, the administration of *Galega officinalis* extract caused a significant decrease in leukocyte proliferation, which is otherwise very high under diabetes. However, a more detailed analysis showed that despite the overall growth of leukocyte proliferation under diabetes mellitus, the abundance of not all leukocyte types increases in the bone marrow [38]. In particular, under diabetes a reduction in the number of myeloblasts was shown, with the following decrease of juvenile and staff neutrophils. By contrast, lymphoblast numbers increased. Interestingly, the number of lymphocytes in the bone marrow does not undergo significant changes, potentially because immature lymphocytes leave the bone marrow towards the bloodstream. Since the non-alkaloid fraction of *Galega officinalis* extract can regulate the proliferative activity of leukocyte precursors, it is able to influence on the content of different types of leukocytes. *Galega officinalis* extract administration causes a decline in lymphoblasts and segmented granulocytes number, as well as an increase in numbers of lymphocytes and juvenile and staff granulocytes in the bone marrow of animals with diabetes mellitus. It has been proposed that this effect is due to the extract's ability to regulate the tumor necrosis factor α (TNF-α) content, the amount of which significantly increases in diabetes mellitus [38].

Furthermore, the revealed influence of *Galega officinalis* extract on the proliferative activity of leukocytes may relate to the presence of inositol [50], fatty acids [51, 52], especially α-linolenic acid [53–55], flavonoids [56–59], phytol [60], squalene [61], campesterol, and stigmasterol [62] as well as α-amyrin [38, 63].

3.3 Influence of *Galega officinalis* on functional state of leukocytes and their antioxidant-prooxidant balance

In diabetes, abnormal immune response manifests itself not only in the imbalance in the process of leukocytes proliferation but also in the disruption of these cells' functional activity. The main effectors of the inflammatory process are phagocytes [64]. The effectiveness of phagocytic response is largely determined by the nature and intensity of its initial stage—chemotaxis. However, because of its complexity, chemotaxis is one of the most vulnerable forms of neutrophil reactivity [65]. Therefore, the impairment of the functional capacity of phagocytes and other immunocytes is associated with the pathology of movement of these cells. The main mechanism that allows cell motility is actin polymerization, as it underlies in the formation of stress fibrils, lamellipodia, and filopodia [66].

In animals with diabetes, the non-alkaloid fraction of *Galega officinalis* extract causes a decrease in filamentous actin (F-actin) content; this can testify about the reduction in the formation of short pseudopodia on the leukocytes surface. These data indicate that the use of this extract reduces the change in the structural and functional properties of leukocytes, as well as decrease of leukocyte pre-activated state [67]. It is possible that the extract-induced decrease in actin polymerization

might regulate integrin-dependent interaction with vascular endothelium necessary for leukocytes penetration through the blood vessel wall during inflammatory processes [68].

F-actin is represented by two pools: (1) long microfilaments (the constitutive fraction of cytoskeleton) located near the cell membrane and reaching towards the center of the cell and (2) short microfilaments located in the submembrane cortical network. Short filaments form a very dynamic fraction, since they are the first ones to initiate polymerization of actin membrane filaments at the time of leukocytes activation [69]. Along with F-actin high content in blood leukocytes in diabetes mellitus condition, the process of its polymerization is intensified with the formation of fraction of short actin filaments. The source of monomers for this polymerization is, to a large extent, products of cytoskeleton filaments depolymerization and, to a lesser extent, the cellular pool of monomeric actin. The increase in actin polymerization may be due to an increase in the phosphatidylinositol amount observed in diabetes mellitus [70]. These cellular messengers may act as inhibitors of phosphorylation of actin regulatory proteins that affect the redistribution of actin filaments and reduce the content of cytoskeleton actin filaments and proportionally increase the level of actin in the short filaments and monomers fractions [71].

The administration of the non-alkaloid fraction of *Galega officinalis* extract in leukocytes of animals with diabetes causes a pronounced depolymerization of short actin filaments. It is accompanied by the formation of actin monomers and their polymerization to a fraction of cytoskeleton filaments. *Galega*-induced changes in actin cytoskeleton organization of leukocytes under prolonged hyperglycemia are probably due to a decrease in the pre-activated state of leukocytes. This effect is mainly achieved by a decrease in the intensity of activation and translocation of the phosphatidylinositol-3′-kinase regulatory subunit in the cytoskeleton sites [68, 72]. Reduced amount of phosphatidylinositol-3′-kinase reaction products (phosphatidylinositol-3,4-diphosphate and phosphatidylinositol-1,3,4-triphosphate) in the cell results in association of the CAP protein with actin filaments, resulting in inhibition of actin polymerization [71].

As mentioned above, diabetes mellitus type 1 is characterized by pre-activated state of leukocytes. This state is associated with the structural and functional rearrangement of the receptor apparatus of these cells. Often, such alterations are realized through changes in the structure of surface glycoproteins that contain sialic acid [73]. In diabetes, N-acetyl-β,D-glucosamine residues are exposed to a greater degree compared to healthy subjects, while the exposure of sialic acids linked by α2→3 and α2→6-glycoside bonds to subterminal residues (β, D-galactose, or N-acetylgalactosamine) decreases. Quantitative redistribution of glycoconjugates in leukocyte membranes leads to the modification of signaling networks involved in intercellular interactions, as well as, to the disruption of the aggregation and adhesiveness of these cells [67]. Activation of membrane-bound neuraminidases in diabetes mellitus leads to a decrease in the total level of sialic acids on the cell membrane. Desialylation is accompanied by increased content of subterminal monosaccharide—β, D-galactose. Galactose-containing glycoproteins regulate leukocyte migration during the inflammatory process, accompanied by a dynamic rearrangement of actin cytoskeleton [74].

The non-alkaloid fraction of *Galega officinalis* extract normalizes the content and structures of the glycoproteins' carbohydrate determinants that form leukocytes' glycocalyx.

Reduction in N-acetyl-β,D-glucosamine residue content upon *Galega officinalis* administration is important to restore normal leukocyte function. Normalized content of such receptors indicates completion of leukocytes pre-activation. It is known that N-acetyl-β,D-glucosamine-containing glycoproteins include a receptor

for N-formyl-methionyl-leucyl-phenylalanine, which stimulates a respiratory burst in neutrophil granulocytes by activating NADPH oxidase [75]. Also, N-acetyl-β,D-glucosamine-containing glycoconjugates are involved in the adhesion of leukocytes to the endothelium during inflammation (through cell surface receptor macrophage-1 antigen or complement receptor 3, which mediates the interaction of neutrophil granulocytes with intercellular adhesion molecule-1) [75]. Thus, the normalization of the receptor content, which has N-acetyl-β,D-glucosamine in its structure, improves the cell's response to extracellular stimuli with a corresponding restoration of the functional state of leukocytes.

Under streptozotocin-induced diabetes, the administration of the non-alkaloid fraction of *Galega officinalis* extract increases the content of α(2→3)-bond sialic acids to physiological levels. It is possible that this effect is due to the influence of the extract's biologically active substances on the activity of enzymes involved in the cleavage or transfer of sialic acid residues (neuraminidase and trans-sialidase) [67, 76]. Glycoproteins that contain sialic acids are structural components of the leukocyte co-receptor complex CD3, which is present in all mature T-lymphocytes and is involved in their activation. It can be assumed that the use of *Galega officinalis* may lead to the restoration of the structure of carbohydrate determinants of the glycoprotein subunit CD3-γ or CD3-ε in the CD3 co-receptor. This in turn inhibits the attenuation of T cells maturation and, as a consequence, prevents the development of the immune deficiency [77–79].

Consequently, receptor apparatus restoration by *Galega officinalis* extract determines the normalization of the cells' response to extracellular signals, which ultimately leads to the reorganization of actin cytoskeleton elements. However, the leukocyte migration, and therefore the state of actin cytoskeleton, depends on the presence of adhesion molecules on leukocyte surface and on the presence of chemokines. One of these chemokines is TNF-α, a pleiotropic pro-inflammatory cytokine. Through the activation of various signaling cascades, it regulates cell proliferation, differentiation, migration, and apoptosis [80, 81]. An increase in cytokine concentrations under diabetes [38, 67] stimulates leukocyte actin polymerization. TNF-α induces a brief increase in polymerized actin content by activating the Rho/ROCK (Rho-related protein kinase) signaling pathway in neutrophils. The activation of the Rho/ROCK signaling pathway leads to the reorganization of the neutrophil cytoskeleton inducing the formation of stress fibers [82–84]. *Galega* extract decreases TNF-α content to physiological levels. This effect is believed to be related to the presence of anti-inflammatory compounds, including flavonoids, methyl ester of linolenic acid, and α-amyrin [67].

Thus, the non-alkaloid fraction of *Galega officinalis* extract reduces leukocyte pre-activation by acting both on cellular receptor apparatus and on chemokine content in the medium. Reducing diabetes-induced leukocytes pre-activated state by *Galega* extract can significantly improve these cells' functional state. One of the most important functional properties of neutrophils is their bactericidal action. It has been discovered that *Galega officinalis* greatly improved the microbe killing properties of cells. In particular, the non-alkaloid fraction of *Galega officinalis* extract causes a decrease in neutrophils myeloperoxidase content, whereas in conditions of diabetes, the content of this enzyme increases [38, 85]. Inhibition of myeloperoxidase production by neutrophils can play an important role in the prevention of vascular damage mediated by leukocytes. It is known that the excessive amount of myeloperoxidase can cause damage of the blood vessel walls by producing strong oxidants (HOCl and HOBr) or by nitration of the tyrosine residues in proteins. Altogether this can eventually result in cardiovascular diseases [86, 87]. It has been proposed that such inhibiting effect of *Galega officinalis* extract may be due to the synergistic action of phytol, flavonoids, squalene, phytosterols, and amyrin [38].

Along with the decrease in the content of myeloperoxidase, the non-alkaloid fraction of the *Galega officinalis* extract also reduces the content of cationic proteins [38] that mediate the killing of a variety of microorganisms through ion pore formation in their membranes [88]. The latter effect is associated with the presence of flavonoids in the extract [38], because these compounds are able to inhibit cationic protein secretion [89].

Thus, the use of alkaloid-free *Galega officinalis* extract for the treatment of diabetes leads to the restoration of functional properties of leukocytes, as indicated by the reconstitution of glycoconjugate receptors on leukocyte membranes, normalization of the ratio of polymerized and unpolymerized actin, as well as restoration of bactericidal properties of these cells.

Diabetes is accompanied by neutrophil malfunction caused, to a large extent, by the development of oxidative-nitrative stress [90]. Oxidative stress leads to the activation of immunocompetent blood cells and their aggregation and adhesion. Further, an increase in the synthesis of arachidonic acid and its metabolites, cytokines, oxygen radicals, and secretion of lysosomal enzymes take place in activated leukocytes. Altogether, it ultimately leads to the development of atherosclerosis [91].

Due to the presence of a large number of biologically active substances with a potential antioxidant effect in the non-alkaloid fraction of *Galega officinalis* extract, it is possible to use this extract as a potential source of antioxidants. Indeed, under diabetes mellitus, the non-alkaloid fraction of *Galega officinalis* extract causes a significant reduction in ROS content in leukocytes, which is otherwise elevated in the pathology [92]. Reduction of ROS generation by leukocytes may be due to the influence of *Galega* extract on the activity of the three main enzymatic systems responsible for generation ROS: membrane-bound NADPH oxidase, peroxidase—myeloperoxidase in neutrophils and eosinophil peroxidase in eosinophils, as well as NO synthase. Indeed, a decrease in the content of myeloperoxidase in polymorphonuclear leukocytes [38] and reduction of the total activity of NO synthase was confirmed [93]. In addition to decreasing the activity of ROS synthesis enzymatic systems, the non-alkaloid extract of *Galega officinalis* significantly reduces the processes of protein and lipid oxidative modification. This effect is due to a decrease in total ROS content and NO stable metabolites (nitrite and nitrate anions), with the corresponding termination of biosubstrate oxidation by free radicals. Reduction of oxidative modified proteins and lipids stops the chain reaction of oxidative-nitric stress in conditions of diabetes and confirms the antioxidant effect of the *Galega officinalis* extract [38, 93].

The negative action of ROS in the body is counterbalanced by an antioxidant system, whose functioning is aimed at neutralizing free radicals, as well as repairing damages caused by them [94]. However, in conditions of oxidative-nitrative stress, which is largely activated during diabetes, antioxidant system of blood cells cannot fully implement its protective and adaptive mechanisms. The abnormal functioning of the immune system is evident from a decrease in the superoxide dismutase, catalase, and glutathione peroxidase activity in leukocytes. Under diabetes, the non-alkaloid fraction *of Galega officinalis* extract has a protective effect on the key components of the antioxidant defense system, causing a significant increase in superoxide dismutase and catalase activities [92]. Restoration of antioxidant defense enzymes activity by biologically active substances may be caused by inhibition of the glycosylation of these enzymes, mediated by the hypoglycemic effect of the extract. The increased activity of the antioxidant enzymes is in line with the observed suppression of the formation of oxygen and nitrogen reactive forms, as well as protein and lipid oxidation [38, 93].

The protective effect of the non-alkaloid fraction of *Galega officinalis* extract on blood cells can be explained by its ability to regulate the prooxidant-antioxidant

balance by means of scavenging free radicals and preventing the inhibition of key components of enzymatic antioxidant system. The main active ingredients of the extract that exhibit antioxidant properties are phytol, showing its properties due to its hydroxyl group [95] and, flavonoids, serving as a traps for electrons and free radicals and thus suppressing the chain reactions of free radical biosubstrate oxidation [38, 89, 93]. Also, α-amyrin [43] and α-linoleic acid [46] possess pronounced antioxidant activities.

3.4 *Galega officinalis* prevents leukocytes apoptosis induced by diabetes mellitus

The development of diabetes mellitus is accompanied by a significant intensification of oxidative-nitrative stress, resulting in the formation of substances with a strong proapoptotic effect. Especially sensitive to such substances are blood cells, including leukocytes. The response of immune cells to antigenic stimuli, as well as the nature, dynamics, and duration of the immune response and immunological tolerance formation are partially regulated through programmed cell death [96]. The non-alkaloid fraction of *Galega officinalis* extract causes inhibition of DNA fragmentation, which is a biochemical marker of apoptosis [97].

Other studies have shown that the use of the non-alkaloid fraction of *Galega officinalis* extract in animals with diabetes leads to a reduction of lymphocytes with features of apoptosis, in particular to reduction of phosphatidylserine (PS) residue translocation from the inner to the outer side of the membrane [38]. Changes in the intensity of lymphocyte apoptosis may be due to the effect of extract on the content of TNF-α. It is known that TNF-α reacts with the so-called death receptors and activates procaspases that trigger the apoptotic cascade [98]. Thus, a decrease in TNF-α content might suggest that one of the mechanisms by which *Galega officinalis* inhibits apoptosis in immunocompetent cells is by suppressing the extrinsic, or death receptor, apoptosis pathway [38].

Another evidence for the activation of the extrinsic apoptosis pathway under diabetes is exposure on leukocytes' immature membrane epitopes with modified sialic acid content. It takes place in response to the loss of surface membrane during cytoplasmic membrane blebbing [99]. The administration of *Galega officinalis* extract to diabetic animals causes an increase in the content of sialic acid residues linked by α(2→3) and α(2→6) glycosidic bonds with the subterminal surface glycoconjugate residues of rat leukocytes [75].

On the other hand, it has been found that *Galega officinalis* is able to regulate the processes of the intrinsic (mitochondrial) pathway of apoptosis. In particular, it reduces the levels of the apoptosis regulatory proteins p53 and Bcl-2 [75, 97]. It is known that cell damage results in p53 translocation from the cytoplasm into the mitochondria [100]. In the mitochondria this protein undergoes rapid enzymatic de-ubiquitination that yields an active form which interacts with BH4 domain of antiapoptotic proteins Bcl-XL and Bcl-2 [100]. Binding to antiapoptotic proteins induces the release and activation of proapoptotic proteins Bax and Bid. Such interactions lead to the release of cytochrome c and induction of apoptosis [101, 102]. At the same time, *Galega officinalis* in leukocytes regulates the content of Bcl-2, a protein that inhibits both p53-dependent and p53-independent pathways of apoptosis. Reduction of this protein content promotes the formation of ion channels in mitochondria membrane, thus stabilizing the mitochondrial cytochrome c oxidase and regulating the activation of proteins that are involved in apoptosis [75, 97].

Another significant confirmation of *Galega officinalis* antiapoptotic action is the reduction of the content of PARylated proteins in leukocytes under diabetes [75].

This indicates a decrease in DNA damage with the corresponding inhibition of DNA repair complex (base excision repair in response to single-stranded DNA breaks and nucleotide excision repair), which includes poly (ADP-ribose) polymerase enzyme [103]. Thus, *Galega*-induced decrease in protein PARylation could stem from inhibition of poly (ADP-ribose) polymerase activity, which can be assumed to prevent ribosylation of a number of proteins, including glyceraldehyde-3-phosphate dehydrogenase. In the presence of excess glucose, this results in inactivation of the polyol and hexosamine pathways, thereby preventing the accumulation of products and precursors of nonenzymatic glycosylation and activation of protein kinase C. As the final result, this leads to the inhibition of oxidative-nitric stress manifestations and prevents the occurrence of chronic diabetic lesions [75].

The established antiapoptotic effect of *Galega officinalis* extract is mediated by sugar-reducing, antioxidant, and anti-inflammatory properties of its components. In particular, the composition of the extract revealed a number of compounds that have potentially hypoglycemic (phytol, ethyl ester of palmitic acid, campesterol, stigmasterol, and quinazoline derivatives), antioxidant (phytol, flavonoids, vitamin E), and anti-inflammatory (flavonoids, methyl ester of linolenic acid, α-amyrin) effects [38].

4. Conclusions

Metformin has become widely used in the treatment of diabetes mellitus type 2 over the last period of time. This is due to the fact that metformin, along with its hypoglycemic effect, has the potential to modulate the functioning of immunocompetent blood cells. Metformin transiently inhibits NADH:ubiquinone oxidoreductase of the mitochondrial electron transport chain. This inhibition leads to the activation of the energy sensor 5′-AMP-activated protein kinase. The activation of this enzyme results in a whole range of metabolic changes in the immunocompetent cells. Metformin is able to regulate the processes of bone marrow cell proliferation, affect the functional activity, and regulate the apoptosis processes of immunocompetent cells.

To date, practically all mechanisms of therapeutic influence of metformin are well described. Instead, the plant from which this biguanide was first obtained somewhat become underestimated. Under diabetes mellitus type 1, the non-alkaloid fraction of *Galega officinalis* possesses pronounced hypoglycemic effect. The non-alkaloid fraction of *Galega officinalis* normalizes the leukocyte proliferation processes by restoring the neutrophils bone marrow pool and reducing the lymphoblasts number. This extract affects the functional state of immunocompetent cells in blood, leading to quantitative redistribution and structural alterations of carbohydrate determinants in leukocyte membranes, reorganization of actin cytoskeleton, as well as affecting the bactericidal function of neutrophils. Furthermore, non-alkaloid fraction of *Galega officinalis* predetermines the suppression of leukocyte to genetically programmed death. The multifactorial effect of *Galega officinalis* extract under diabetes may be, on the one hand, due to its potent hypoglycemic effect, and, on the other hand, due to its ability to regulate the prooxidant-antioxidant balance by scavenging free radicals and preventing the inhibition of key enzymatic components of the antioxidant defense system.

Conflict of interest

The authors declare no conflict of interest.

IntechOpen

Author details

Mariia Nagalievska*, Halyna Hachkova and Nataliia Sybirna
Department of Biochemistry, Faculty of Biology, Ivan Franko National University of Lviv, Lviv, Ukraine

*Address all correspondence to: khmarija@gmail.com

References

[1] Bajpai A, Tilley DG. The role of leukocytes in diabetic cardiomyopathy. Frontiers in Physiology. 2018;**9**:1547. DOI: 10.3389/fphys.2018.01547

[2] Takeda Y, Asao H, Wakabayashi I. An analysis of the intracellular signal transduction of peripheral blood leukocytes in animal models of diabetes using flow cytometry. Methods in Molecular Biology. 1916;**2019**:177-193. DOI: 10.1007/978-1-4939-8994-2_17

[3] de Souza Prestes A, Dos Santos MM, Ecker A, de Macedo GT, Fachinetto R, Bressan GN, et al. Methylglyoxal disturbs the expression of antioxidant, apoptotic and glycation responsive genes and triggers programmed cell death in human leukocytes. Toxicology In Vitro. 2019;**55**:33-42. DOI: 10.1016/j.tiv.2018.11.001

[4] Pan S, Zhou S, Gao S, Yu Z, Zhang S, Tang M, et al. New perspectives on how to discover drugs from herbal medicines: CAM's outstanding contribution to modern therapeutics. Evidence-based Complementary and Alternative Medicine. 2013;**2013**:627375. DOI: 10.1155/2013/627375

[5] Shokri F, Shokoohi M, Niazkar HR, Abadi ARR, Kalarestaghi H, Ahin M. Investigation the spermatogenesis and testis structure in diabetic rats after treatment with *Galega officinalis* extract. Crescent Journal of Medical and Biological Sciences. 2019;**6**(1):31-36

[6] Lupak M, Khokhla M, Hachkova G, Shulga O, Sheglova N, Vildanova R, et al. Application of biogenic surfactants for stabilization of alkaloid-free fraction isolated from *Galega officinalis* extract. Biologica Studia. 2015;**9**(1):25-36. DOI: 10.30970/sbi.0901.397

[7] Khokhla M, Kleveta G, Kotyk A, Skybitska M, Chajka Y, Sybirna N. Sugar-lowering effects of *Galega officinalis* L. Annales Universitatis Mariae Curie-Sklodowska, Sectio DDD: Pharmacia. 2010;**23**(4):177-182

[8] Khodadadi S. Administration of *Galega officinalis* in experimental and clinical investigations; A narrative review. Annals of Research in Antioxidants. 2016;**1**(1):e03

[9] Ursini F, Russo E, Pellino G, D'Angelo S, Chiaravalloti A, De Sarro G, et al. Metformin and autoimmunity: A "new deal" of an old drug. Frontiers in Immunology. 2018;**9**:1236. DOI: 10.3389/fimmu.2018.01236

[10] Queiroz EAIF, Puukila S, Eichler R, Sampaio SC, Forsyth HL, Barbosa AM, et al. Metformin induces apoptosis and cell cycle arrest mediated by oxidative stress, AMPK and FOXO3a in MCF-7 breast cancer cells. PLoS One. 2014;**9**(5):e98207. DOI: 10.1371/journal.pone.0098207

[11] de Oliveira S, Houseright RA, Graves AL, Golenberg N, Korte BG, Miskolci V, et al. Metformin modulates innate immune-mediated inflammation and early progression of NAFLD-associated hepatocellular carcinoma in zebrafish. Journal of Hepatology. 2019;**70**:710-721. DOI: 10.1016/j.jhep.2018.11.034

[12] Bae H, Zmijewski JW, Deshane JS, Tadie J, Chaplin DD, Takashima S, et al. AMP-activated protein kinase enhances the phagocytic ability of macrophages and neutrophils. The FASEB Journal. 2011;**25**(12):4358-4368. DOI: 10.1096/fj.11-190587

[13] Park DW, Jiang S, Tadie J, Stigler WS, Gao Y, Deshane J, et al. Activation of AMPK enhances neutrophil chemotaxis and bacterial killing. Molecular Medicine.

2013;**19**:387-398. DOI: 10.2119/molmed.2013.00065

[14] Pollak M. The effects of metformin on gut microbiota and the immune system as research frontiers. Diabetologia. 2017;**60**:1662-1667. DOI: 10.1007/s00125-017-4352-x

[15] Zhang Q, Tang W, Deater M, Phan N, Marcogliese AN, Li H, et al. Metformin improves defective hematopoiesis and delays tumor formation in Fanconi anemia mice. Blood. 2016;**128**(24):2774-2784. DOI: 10.1182/blood2015-11-683490

[16] Xu G, Wu H, Zhang J, Li D, Wang Y, Wang Y, et al. Metformin ameliorates ionizing irradiation-induced long-term hematopoietic stem cell injury in mice. Free Radical Biology and Medicine. 2015;**87**:15-25. DOI: 10.1016/j.freeradbiomed.2015.05.045

[17] Orio F, Manguso F, Di Biase S, Falbo A, Giallauria F, Labella D, et al. Metformin administration improves leukocyte count in women with polycystic ovary syndrome: A 6-month prospective study. European Journal of Endocrinology. 2007;**157**:69-73. DOI: 10.1530/EJE-07-0133

[18] Kebir DE, Filep JG. Role of neutrophil apoptosis in the resolution of inflammation. The Scientific World Journal. 2010;**10**:1731-1748. DOI: 10.1100/tsw.2010.169

[19] Isoda K, Young JL, Zirlik A, MacFarlane LA, Tsuboi N, Gerdes N, et al. Metformin inhibits proinflammatory responses and nuclear factor-B in human vascular wall cells. Arteriosclerosis, Thrombosis, and Vascular Biology. 2006;**26**:611-617. DOI: 10.1161/01.ATV.0000201938.78044.75

[20] Nath N, Khan M, Paintlia MK, Hoda MN, Giri S. Metformin attenuated the autoimmune disease of the central nervous system in animal models of multiple sclerosis. The Journal of Immunology. 2009;**182**:8005-8014. DOI: 10.4049/jimmunol.0803563

[21] Han J, Li Y, Liu X, Zhou T, Sun H, Edwards P, et al. Metformin suppresses retinal angiogenesis and inflammation in vitro and in vivo. PLoS One. 2018;**13**(3):e0193031. DOI: 10.1371/journal.pone.0193031

[22] Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circulation Research. 2010;**107**:1058-1070. DOI: 10.1161/CIRCRESAHA.110.223545

[23] Foretz M, Guigas B, Bertrand L, Pollak M, Viollet B. Metformin: From mechanisms of action to therapies. Cell Metabolism. 2014;**20**(6):953-966. DOI: 10.1016/j.cmet.2014.09.018

[24] Victor VM, Rovira-Llopis S, Banuls C, Diaz-Morales N, Castello´ R, Falcon1 R, et al. Effects of metformin on mitochondrial function of leukocytes from polycystic ovary syndrome patients with insulin resistance. European Journal of Endocrinology. 2015;**173**:683-691. DOI: 10.1530/EJE-15-0572

[25] Bonnefont-Rousselot D, Raji B, Walrand S, Garde's-Albert M, Jore D, Legrand A, et al. An intracellular modulation of free radical production could contribute to the beneficial effects of metformin towards oxidative stress. Metabolism. 2003;**52**(5):586-589. DOI: 10.1053/meta.2003.50093

[26] Onaran I, Guven GS, Ozdas SB, Kanigur G, Vehid S. Metformin does not prevent DNA damage in lymphocytes despite its antioxidant properties against cumene hydroperoxide-induced oxidative stress. Mutation Research. 2006;**611**:1-8. DOI: 10.1016/j.mrgentox.2006.06.036

[27] Lee SC, Pervaiz S. Apoptosis in the pathophysiology of diabetes

mellitus. The International Journal of Biochemistry & Cell Biology. 2007;**39**(3):497-504. DOI: 10.1016/j.biocel.2006.09.007

[28] Kanigür-Sultuybek G, Ozdas ŞB, Curgunlu A, Tezcan V, Onaran I. Does metformin prevent short term oxidant-induced DNA damage? In vitro study on lymphocytes from aged subjects. Journal of Basic and Clinical Physiology and Pharmacology. 2007;**18**(2):129-140. DOI: 10.1515/jbcpp.2007.18.2.129

[29] Ullah I, Ullah N, Naseer MI, Lee HY, Kim MOK. Neuroprotection with metformin and thymoquinone against ethanol-induced apoptotic neurodegeneration in prenatal rat cortical neurons. BMC Neuroscience. 2012;**13**:11. DOI: 10.1186/1471-2202-13-11

[30] de la Rosa LC, Vrenken TE, Buist-Homan M, Faber KN, Moshage H. Metformin protects primary rat hepatocytes against oxidative stress-induced apoptosis. Pharmaceutical Research & Perspectives. 2015;**3**(2):e00125. DOI: 10.1002/prp2.125

[31] Wiernsperger N. Metformin as a cellular protector; a synoptic view of modern evidences. Journal of Nephropharmacology. 2015;**4**(1):31-36

[32] Cheki M, Ghasemi MS, Rashnoudi AMR, Majd NE. Metformin attenuates cisplatin-induced genotoxicity and apoptosis in rat bone marrow cells. Drug and Chemical Toxicology. 2019;**9**:1-8. DOI: 10.1080/01480545.2019.1609024

[33] Wang Z, Liu X, Wang M, Jiang G, Qiu T, Chen Z, et al. Metformin attenuated the inflammation after renal ischemia/reperfusion and suppressed apoptosis of renal tubular epithelial cell in rats. Acta Cirúrgica Brasileira. 2015;**30**(9):617-623. DOI: 10.1590/S0102-865020150090000006

[34] Bromfield ESP, Cloutier S, Robidas C, Thi TVT, Darbyshire SJ. Invasive *Galega officinalis* (Goat's rue) plants in Canada form a symbiotic association with strains of *Neorhizobium galegae* sv. officinalis originating from the Old World. Ecology and Evolution. 2019;**00**:1-6. DOI: 10.1002/ece3.5266

[35] Karakas FP, Turker AU, Karakas A, Mshvildadze V. Cytotoxic, anti-inflammatory and antioxidant activities of four different extracts of *Galega officinalis* L (Goat's rue). Tropical Journal of Pharmaceutical Research. 2016;**15**(4):751-757. DOI: 10.4314/tjpr.v15i4.12

[36] Khokhla M, Kleveta H, Lupak M, Kaniuka O, Chaika IA, Skybitska M, et al. The research of *Galega officinalis* extract components. Visnyk of L'viv University. Biological Series. 2013;**62**: 55-60 (in Ukrainian)

[37] Alqahtani N, Khan WA, Alhumaidi MH, Ahmed YA. Use of glycated hemoglobin in the diagnosis of diabetes mellitus and pre-diabetes and role of fasting plasma glucose, oral glucose tolerance test. International Journal of Preventive Medicine. 2013;**4**(9):1025-1029

[38] Nagalievska M, Sabadashka M, Hachkova H, Sybirna N. *Galega officinalis* extract regulate the diabetes mellitus related violations of proliferation, functions and apoptosis of leukocytes. BMC Complementary and Alternative Medicine. 2018;**18**(1):4. DOI: 10.1186/s12906-017-2079-3

[39] Elmazar M, El-Abhar HS, Schaalan MF, Schaalan MF, Farag NA. *P*hytol/phytanic acid and insulin resistance: Potential role of phytanic acid proven by docking simulation and modulation of biochemical alterations. PLoS One. 2013;**8**(1):1-10. DOI: 10.1371/journal.pone.0045638

[40] Sarkodie J, Fleischer T, Edoh DA, Dickson RA, Mensah MLK, Annan K, et al. Antihyperglycaemic activity of ethanolic extract of the stem of *Adenia lobata* Engl (Passifloraceae). International Journal of Pharmaceutical Sciences and Research. 2013;**4**(4):1370-1377

[41] Lee YM, Haastert B, Scherbaum W, Hauner H. A phytosterol-enriched spread improves the lipid profile of subjects with type 2 diabetes mellitus. A randomized controlled trial under free-living conditions. European Journal of Nutrition. 2003;**42**:111-117. DOI: 10.1007/s00394-003-0401-y

[42] Tanaka M, Misawa E, Ito Y, Habara N, Nomaguchi K, Yamada M, et al. Identification of five phytosterols from Aloe vera gel as antidiabetic compounds. Biological & Pharmaceutical Bulletin. 2006;**29**(7):1418-1422. DOI: 10.1248/bpb.29.1418

[43] Santos FA, Frota JT, Arruda BR, de Melo TS, da Silva AA, Brito GA, et al. Antihyperglycemic and hypolipidemic effects of α,β-amyrin, a triterpenoid mixture from *Protium heptaphyllum* in mice. Lipids in Health and Disease. 2012;**11**:98. DOI: 10.1186/1476-511X-11-98

[44] Nam Kyu Lee (SuwonSi, KR), Jun Won Lee (Gunpo-Si, KR), Sukho Lee (Suwon-Si, KR), Guang-Jin Im (Ansan-Si, KR), Hye Young Han (Seoul, KR), Tae Kon Kim (Suwon-Si, KR), Yong Hyuk Kim (Suwon-Si, KR), Wie-Jong Kwak (Seoul, KR), Wie-Jong Kwak (Seoul, KR), Sang Woong Kim (Daejeon, KR), Joohun Ha (Seoul, KR), Eon Kyum Kim (Daejeon, KR), Jung Kyu Lee (Daejeon, KR), Choong Yeul Yoo (Daejeon, KR), Dae Yeon Lee (Daegu, KR). Quinazoline derivatives for the treatment and prevention of diabetes and obesity. Patent application number: 20080207614

[45] Leclerc I, Woltersdorf WW, da Silva Xavier G, Rowe RL, Cross SE, Korbutt GS, et al. Metformin, but not leptin, regulates AMP-activated protein kinase in pancreatic islets: Impact on glucose-stimulated insulin secretion. American Journal of Physiology-Endocrinology and Metabolism. 2004;**286**(6):E1023-E1031. DOI: 10.1152/ajpendo.00532.2003

[46] Rodriguez-Leyva D, Dupasquier CM, McCullough R, Pierce GN. The cardiovascular effects of flaxseed and its omega-3 fatty acid, alphalinolenic acid. The Canadian Journal of Cardiology. 2010;**26**(9):489-496. DOI: 10.1016/s0828-282x(10)70455-4

[47] Lontchi-Yimagou E, Sobngwi E, Matsha TE, Kengne AP. Diabetes mellitus and inflammation. Current Diabetes Reports. 2013;**13**(3):435-444. DOI: 10.1007/s11892-013-0375-y

[48] Erbağcia AB, Tarakçioğlua M, Coşkunb Y, Sivaslib E, Namidurua ES. Mediators of inflammation in children with type I diabetes mellitus: Cytokines in type I diabetic children. Clinical Biochemistry. 2001 Nov;**34**(8):645-650. DOI: 10.1016/S0009-9120(01)00275-2

[49] Khokhla MR, Kleveta GY, Chajka YP, Skybitska MI, Sybirna NO. Cytological and biochemical characteristics of rats' peripheral blood under the condition of experimental diabetes mellitus type 1 and *Galega officinalis* admission. Biological Studies. 2012;**6**(1):37-46. DOI: 10.30970/sbi.0601.189

[50] Deliliers GL, Servida F, Fracchiolla NS, Ricci C, Borsotti C, Colombo G, et al. Effect of inositol hexaphosphate (IP6) on human normal and leukaemic haematopoietic cells. British Journal of Haematology. 2002;**117**(3):577-587. DOI: 10.1046/j.1365-2141.2002.03453.x

[51] Thies F, Nebe-von-Caron G, Powell JR, Yaqoob P, Newsholme EA, Calder PC. Dietary supplementation

with g-linolenic acid or fish oil decreases T lymphocyte proliferation in healthy older humans. The Journal of Nutrition. 2001;**131**(7):1918-1927. DOI: 10.1093/jn/131.7.1918

[52] Yaqoob P, Newsholme EA, Calder PC. The effect of fatty acids on leucocyte subsets and proliferation in rat whole blood. Nutrition Research. 1995;**15**(2):279-287. DOI: 10.1016/0271-5317(95)92592-8

[53] Sravan Kumar G, Das UN, Vijay Kumar K, Madhavi N, Das NP, Tan BKH. Effect of n-6 and n-3 fatty acids on the proliferation of human lymphocytes and their secretion of TNF-α and IL-2 in vitro. Nutrition Research. 1992;**12**(7):815-823

[54] Calder PC, Yaqoob P, Thies F, Wallace FA, Miles EA. Fatty acids and lymphocyte functions. British Journal of Nutrition. 2002;**87**(1):S31-S48. DOI: 10.1079/BJN2001455

[55] Caughey GE, Mantzioris E, Gibson RA, Cleland LG, James MJ. The effect on human tumor necrosis factor A and interleukin 1 production of diets enriched in n-3 fatty acids from vegetable oil or fish oil. American Journal of Clinical Nutrition. 1996;**63**(1):16-22. DOI: 10.1093/ajcn/63.1.116

[56] Hirano T, Oka K, Kawashima E, Akiba M. Effects of synthetic and naturally occurring flavonoids of mitogen-induced proliferation of human peripheral-blood lymphocytes. Life Sciences. 1989;**45**(15):1407-1411. DOI: 10.1016/0024-3205(89)90028-3

[57] Nijveldt RJ, van Nood E, van Hoorn DEC, Boelens PG, van Norren K, van Leeuwen PAM. Flavonoids: A review of probable mechanisms of action and potential applications. The American Journal of Clinical Nutrition. 2001;**74**:418-425. DOI: 10.1093/ajcn/74.4.418

[58] Saraf S, Singh MA, Saraf A. Flavonoids: A nutritional protection against oxidative and UV induced cellular damages. Pharmacognosy Reviews. 2007;**1**:30-40

[59] Kim HP, Son KH, Chang HW, Kang SS. Anti-inflammatory plant flavonoids and cellular action mechanisms. Journal of Pharmacological Sciences. 2004;**96**: 229-245. DOI: 10.1254/jphs.CRJ04003X

[60] Silva RO, Sousa FBM, Damasceno SRB, Carvalho NS, Silva VG, Oliveira FRMA, et al. Phytol, a diterpene alcohol, inhibits the inflammatory response by reducing cytokine production and oxidative stress. Fundamental & Clinical Pharmacology. 2014;**28**(4):455-464. DOI: 10.1111/fcp.12049

[61] Cárdeno A, Aparicio-Soto M, Montserrat-de la Paz S, Bermudez B, Muriana FJG, Alarcón-de-la-Lastra C. Squalene targets pro- and anti-inflammatory mediators and pathways to modulate over-activation of neutrophils, monocytes and macrophages. Journal of Functional Foods. 2015;**14**:779-790. DOI: 10.1016/j.jff.2015.03.009

[62] Brüll F, Mensink RP, Plat J. Plant sterols: Functional lipids in immune function and inflammation? Clinical Lipidology. 2009;**4**(3):355-365. DOI: 10.2217/clp.09.26

[63] Silva KA, Paszcuk AF, Passos GF, Silva ES, Bento AF, Meotti FC, et al. Activation of cannabinoid receptors by the pentacyclic triterpene α,β-amyrin inhibits inflammatory and neuropathic persistent pain in mice. Pain. 2011;**152**(8):1872-1887. DOI: 10.1016/j.pain.2011.04.005

[64] Rosales C, Juliano RL. Signal transduction by cell adhesion receptors in leukocytes. Journal of Leukocyte

Biology. 1995;**57**(2):189-198. DOI: 10.1002/jlb.57.2.189

[65] Koval'chuk LV, Saĭgitov RT. Chemokines—A new family of cytokines regulating leukocyte migration. Journal of Microbiology, Epidemiology and Immunobiology. 2000;**1**:69-70

[66] Carlier M, editor. Actin-based Motility: Cellular, Molecular and Physical Aspects. Springer; 2010. 435 p

[67] Lupak M, Hachkova H, Khokhla M, Chajka Y, Skybitska M, Sybirna N. Leukocyte actin cytoskeleton reorganization and redistribution of sialylated membrane glycoconjugates under experimental diabetes mellitus and against the administration of the *Galega officinalis* L. extract. Cytology and Genetics. 2017;**51**(3):162-172. DOI: 10.3103/S0095452717030070

[68] Advani A, Marshall S, Thomas T. Increasing neutrophil F-actin corrects CD11b exposure in type 2 diabetes. European Journal of Clinical Investigation. 2004;**34**(5):358-364. DOI: 10.1111/j.1365-2362.2004.01346.x

[69] Hannigan M, Zhan L, Ai Y, Huang CK. Leukocyte-specific gene 1 protein (LSP1) is involved in chemokine KC-activated cytoskeletal reorganization in murine neutrophils in vitro. Journal of Leukocyte Biology. 2001;**69**(3):497-504. DOI: 10.1189/jlb.69.3.497

[70] Sybirna N, Zdioruk M, Brodyak I, Bars'ka M, Vovk O. Activation of the phosphatidylinositol-3′-kinase pathway with lectin induced signal through sialocontaining glycoproteins of leukocyte membranes under type 1 diabetes mellitus. Ukrainskiĭ Biokhimicheskiĭ Zhurnal. 2011;**83**(5):22-31

[71] Kleveta G, Borzęcka K, Zdioruk M, Czerkies M, Kuberczyk H, Sybirna N, et al. LPS induces phosphorylation of actin-regulatory proteins leading to actin reassembly and macrophage motility. Journal of Cellular Biochemistry. 2012;**113**(1):80-92. DOI: 10.1002/jcb.23330

[72] Samstag Y, Eibert SM, Klemke M, Wabnitz G. Actin cytoskeletal dynamics inT-lymphocyte activation and migration. Journal of Leukocyte Biology. 2003;**73**(1):30-48. DOI: 10.1189/jlb.0602272

[73] Sybirna NO, Brodyak IV, Bars'ka ML, Vovk OI. Participation of phosphatidylinositol-3′-kinase in signal transduction through galactosyl-containing glycoprotein receptors of segmentonuclear leukocytes under type 1 diabetes mellitus. Fiziologicheskiĭ Zhurnal. 2012;**58**(6):9-22

[74] Fernández-Rodríguez J, Feijoo-Carnero C, Merino-Trigo A, Páez de la Cadena M, Rodríguez-Berrocal FJ, de Carlos A, et al. Immunohistochemical analysis of sialic acid and fucose composition inhuman colorectal adenocarcinoma. Tumour Biology. 2000;**21**(3):153-164. DOI: 10.1159/000030122

[75] Karlsson A. Wheat gernagglutinin induced NADPH-oxidase in human neutrophils by interaction with mobilizable receptors. Infection and Immunity. 1999;**67**(7):3461-3468

[76] Khokhla M, Kleveta G, Lupak M, Skybitska M, Chajka Y, Sybirna N. The inhibition of rat leukocytes apoptosis under the condition of experimental diabetes mellitus type 1 by *Galega officinalis* L. extract. Current Issues in Pharmacy and Medical Sciences. 2013;**26**(4):393-397. DOI: 10.12923/j.2084-980X/26.4/a.09

[77] Soudais C, de Villartay JP, Le Deist F, Fischer A, Lisowska-Grospierre B. Independent mutations of the human CD3-ε gene resulting in a T cell receptor/CD3 complex

immunodeficiency. Nature Genetics. 1993;**3**(1):77-81. DOI: 10.1038/ng0193-77

[78] Eash S, Tavares R, Stopa EG, Robbins SH, Brossay L, Atwood WJ. Differential distribution of the JC virus receptor-type sialic acid in normal human tissues. The American Journal of Pathology. 2004;**164**(2):419-428. DOI: 10.1016/S0002-9440(10)63132-X

[79] Aiba Y, Kameyama M, Yamazaki T, Tedder TF, Kurosaki T. Regulation of B-cell development by BCAP and CD19 through their binding to phosphoinositide 3-kinase. Blood. 2008;**111**(3):1497-1503. DOI: 10.1182/blood-2007-08-109769

[80] Baud V, Karin M. Signal transduction by tumor necrosis factor and its relatives. Trends in Cell Biology. 2001;**11**(9):372-377. DOI: 10.1016/S0962-8924(01)02064-5

[81] Nakao S, Kuwano T, Ishibashi T, Kuwano M, Ono M. Synergistic effect of TNF-alpha in soluble VCAM-1-induced angiogenesis through alpha 4 integrins. Journal of Immunology. 2003;**170**(11):5704-5711. DOI: 10.4049/jimmunol.170.11.5704

[82] Hahmann C, Schroeter T. Rho-kinase inhibitors as therapeutics: From pan inhibition to isoform selectivity. Cellular and Molecular Life Sciences. 2010;**67**(2):171-177. DOI: 10.1007/s00018-009-0189-x

[83] Riento K, Ridley AJROCKS. Multifunctional kinases in cell behaviours. Nature Reviews. Molecular Cell Biology. 2003;**4**(6):446-456. DOI: 10.1038/nrm1128.

[84] Arita R, Nakao S, Kita T, Kawahara S, Asato R, Yoshida S, et al. A key role for ROCK in TNF-a–mediated diabetic microvascular damage. Investigative Ophthalmology & Visual Science. 2013;**54**(3):2373-2383. DOI: 10.1167/iovs.12-10757

[85] Allen RC, Stephens JT. Myeloperoxidase selectively binds and selectively kills microbes. Infection and Immunity. 2011;**79**(1):474-485. DOI: 10.1128/IAI.00910-09

[86] Gorudko V, Kostevich VA, Sokolov AV, Shamova EV, Buko IV, Konstantinova EE, et al. Functional activity of neutrophils in diabetes mellitus and coronary heart disease: Role of myeloperoxidase in the development of oxidative stress general pathology and pathophysiology. Bulletin of Experimental Biology and Medicine. 2012;**154**(1):23-26. DOI: 10.1007/s10517-012-1865-7

[87] Nicholls SJ, Hazen SL. Myeloperoxidase and cardiovascular disease. Arteriosclerosis, Thrombosis, and Vascular Biology. 2005;**25**:1102-1111. DOI: 10.1161/01.ATV.0000163262.83456.6d

[88] Cruse JM, Lewis RE. Atlas of Immunology. 2nd ed. CRC Press LLC; 2004. 958 p

[89] Middleton E. Biological properties of plant flavonoids: An overview. International Journal of Pharmacognosy. 1996;**34**(5):344-348. DOI: 10.1076/phbi.34.5.344.13245

[90] Lee HB, Ha H, King GL. Reactive oxygen species and diabetic nephropathy. Journal of the American Society of Nephrology. 2003;**14**(3):209-210. DOI: 10.1097/01.ASN.0000077403.06195.D2

[91] Morel O, Pereira B, Averous G, Faure A, Jesel L, Germain P, et al. Increased levels of procoagulant tissue factor-bearing microparticles within the occluded coronary artery of patients with ST-segment elevation myocardial infarction:

Role of endothelial damage and leukocyte activation. Atherosclerosis. 2009;**204**(2):636-641. DOI: 10.1016/j.atherosclerosis.2008.10.039

[92] Lupak MI, Khokhla MR, Hachkova GY, Kanyuka OP, Klymyshyn NI, Chajka YP, et al. The alkaloid-free fraction from *Galega officinalis* extract prevents oxidative stress under experimental diabetes mellitus. Ukranian Biochemical Journal. 2015;**87**(4):78-86. DOI: 10.15407/ubj87.04.078

[93] Lupak MI, Kanyuka OP, Hachkova GY, Chajka YP, Skybitska MI, Sybirna NO. Influence of alkaloid-free fraction of *Galega officinalis* extract on L-arginin/NO system of rats leukocytes under the experimental diabetes mellitus type 1. Medical and Clinical Chemistry. 2014;**16**(3):108-110

[94] Atalay V, Laaksonen DE, Niskanen L. Altered antioxidant enzyme defenses in insulin-dependent diabetic men with increased resting and exercise-induced oxidative stress. Acta Physiologica Scandinavica. 1997;**161**:195-201. DOI: 10.1046/j.1365-201X.1997.00200.x

[95] De Menezes Patrício Santos CC, Salvadori MS, Mota VG, Costa LM, De Almeida AAC, Lopes De Oliveira GA, et al. Antinociceptive and antioxidant activities of phytol in vivo and in vitro models. Neuroscience Journal. 2013;**2013**:1-9. DOI: 10.1155/2013/949452

[96] Hetts SW. To die or not to die: An overview of apoptosis and its role in disease. Journal of the American Medical Association. 1998;**279**(4):300-307. DOI: 10.1001/jama.279.4.300

[97] Khokhla M, Kleveta G, Chajka YA, Skybitska M, Sybirna N. The influence of *Galega officinalis* on rats leukocytes apoptosis under the experimental diabetes mellitus type 1. Visnyk of Lviv University. Biological Series. 2012;**60**:117-125

[98] Deng Y, Ren X, Yang L, Lin Y, Wu X. A JNK-dependent pathway is required for TNFalpha-induced apoptosis. Cell. 2003;**115**(1):61-70. DOI: 10.1016/S0092-8674(03)00757-8

[99] Meesmann HM, Fehr EM, Kierschke S, Herrmann M, Bilyy R, Heyder P, et al. Decrease of sialic acid residues as an eat-me signal on the surface of apoptotic lymphocytes. Journal of Cell Science. 2010;**123**(Pt19):3347-3356. DOI: 10.1242/jcs.066696

[100] Braithwaite A, Royds J, Jackson P. The p53 story: Layers of complexity. Carcinogenesis. 2005 Jul;**26**(7):1161-1169. DOI: 10.1093/carcin/bgi091

[101] Erster S, Mihara M, Kim R. In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation. Molecular and Cellular Biology. 2004;**24**(15):6728-6741. DOI: 10.1128/MCB.24.15.6728-6741.2004

[102] Janicke RU, Sohn D, Schulze-Osthoff K. The dark side of a tumor suppressor: Anti-apoptotic p53. Cell Death and Differentiation. 2008 Jun;**15**(6):959-976. DOI: 10.1038/cdd.2008.33

[103] Schmitz H. Reversible nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase upon serum depletion. European Journal of Cell Biology. 2001;**80**(6):419-427. DOI: 10.1078/0171-9335-00174