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Renal Effects of Goutweed (*Aegopodium podagraria L.*) Preparations and Metformin in Rats with the Disorders of Metabolism Induced by Protamine Sulphate and Atherogenic Diet

Tovchiga OV and Shtrygol' SYu

Department of Pharmacology, National University of Pharmacy, Kharkiv, Ukraine

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***Corresponding author:** Tovchiga Olga Vladimirovna, Department of Pharmacology, National University of Pharmacy, 53 Pushinskaya str., Kharkiv, 61002, Ukraine, Tel: +380-577063069;
E-mail: olga_234@bigmir.net

Abstract

It has been previously shown that the tincture and the extract obtained from *Aegopodium podagraria* L. can be favorably combined with metformin. This study aimed to evaluate the influence of the extract (1 g/kg), the tincture (1 ml/kg) and their combinations with metformin (50 mg/kg) on the excretory renal function in rats receiving the atherogenic diet combined with protamine sulfate during 15 days. The model was characterized by the decline in glomerular filtration rate (GFR) in water loading test, decrease in filtered load of sodium, its proximal and distal transport as well as excretion, the reduction of diuresis, potassium excretion, urine Na^+/K^+ ratio. Metformin decreased creatininemia but led to the further decline in diuresis due to the enhancement in water reabsorption, proteinuria (but not protein excretion) was increased. Goutweed extracts per se and combined with metformin, enhanced water reabsorption, while GFR tended to the increase and diuresis remained unchanged. It normalized the filtered load of sodium and its proximal, but not distal, transport resulting in higher sodium excretion, significantly increased potassium excretion (urine Na^+/K^+ ratio remained reduced). Goutweed tincture normalized sodium and potassium excretion as well as urine Na^+/K^+ ratio, GFR, filtered load and proximal, but not distal, transport of sodium. Water reabsorption was unchanged, diuresis increased. After combined use of the tincture with metformin the renal effects of the latter, but not of the tincture were realized, antiproteinuric effect and minimal activity of alkaline phosphatase in plasma and urine were registered just in this group.

Keywords: Goutweed (*Aegopodium podagraria* L.); Metformin; Dyslipidemia; Kidney; Combined drugs

Introduction

The total number of adult patients with diabetes mellitus was estimated as 422 million in 2014, compared to 108 million in 1980 [1] and the far-reaching global consequences of this disease are unquestionable.

Disorders of lipid metabolism are among the most important factors in the pathogenesis of diabetes mellitus as well as metabolic syndrome and obesity. Among the drugs used to treat these conditions, a special place belongs to metformin, which, in addition to the beneficial influence on the metabolism of carbohydrates and lipids, demonstrates important additional advantages: elimination of endothelial dysfunction and oxidative stress, kidney protection (especially in diabetic nephropathy) as well as possible prolongation of life [2,3]. At the same time, there is an increasing interest in the combined use of herbal drugs and phytochemical constituents with conventional medicines, which can allow reducing the dose of the latter and obtaining favorable concomitant effects [4]. From the other point of view, undesirable results of pharmacokinetic and pharmacodynamic interactions between conventional medicines and substances of herbal origin are possible. For this reason, verification of such combinations efficacy is strongly needed.

It has been shown earlier that it is possible to reduce the effective dose of metformin through its combined administration with the tincture obtained from the aerial part of goutweed (GW, *Aegopodium podagraria* L., Apiaceae). Such phenomenon was established in the glucose tolerance test in the intact rats [5], and in dexamethasone-treated rats the permissive action of the tincture on the normalizing effect of metformin on carbohydrate and lipid metabolism was demonstrated [6]. In both cases the tincture was effective at a dose of 1.0 ml/kg. The tincture itself possesses favorable metabolic effects including antidiabetic activity as well as hepatoprotective, nephroprotective properties and an ability to normalize uric acid metabolism [7-9]. The beneficial pharmacological effects and raw material availability substantiate the use of *A. podagraria* L. for the

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obtaining of drugs and functional foods. In this connection, the methods were proposed for analysis and standardization of GW raw material as well as preparations obtained from it. Hydroxycinnamic acids, flavonoids, polysaccharide-protein complex components, micro- and macroelements were identified in these objects [7,9-11].

Considering the significance of the lipid metabolism disorders in the pathogenesis of diabetes mellitus and other "diseases of civilization", the further studies of the efficacy of GW tincture combination with metformin were conducted on the model of the primary disorders of the lipid metabolism in rats (administration of protamine sulphate as lipoprotein lipase blocker and heparin antagonist against the background of atherogenic diet). GW aerial part water extract was also studied in these experiments because previously it demonstrated an ability to normalize the lipid metabolism [12] as well as the significant nephroprotective and hepatoprotective properties on the models with the different pathogenesis [7,8,13]. It was shown that both GW preparations as well as their combinations with metformin normalized the lipid composition of the liver. The tincture (but not the extract) also demonstrated an ability to increase metformin efficacy in dyslipidemic rats, in which the area under glucose curve in the glucose tolerance test was reduced only after combined administration of these agents [14]. Histological studies showed that after combined administration of GW preparations and metformin their protective activity in regard to the heart structure was maintained, against the background of metformin together with the tincture such activity in regard to the kidney structure was also evident, while there were neither increase in the severity of the liver changes, nor any other signs of toxicity enhancement [15]. In rats treated with the investigated drugs some positive changes were also seen in uric acid metabolism, including xanthine oxidase activity suppression in the liver and the kidneys [16]. Since it is generally accepted that the kidney is among the target organs of diabetes and dyslipidemia, it is expedient to complete the aforesaid data with the results concerning kidney function and water-salt metabolism.

The aim of this study was to evaluate the influence of *A. podagraria* L. tincture and the extract as well as their combinations with metformin on the excretory renal function and water-salt metabolism on the model of the primary disorders of the lipid metabolism in rats.

Materials and Methods

Plant material

The aerial parts of *A. podagraria* L. were collected from natural population in Kharkiv region (Ukraine) in June. Voucher specimens were identified by Ass. Prof. Dr. SI Stepanova and deposited at the Department of Nutriciology and Pharmaceutical Bromatology (National University of Pharmacy, Kharkiv, Ukraine). The herbal raw material was dried at room temperature and powdered using a standard grinding mill to obtain the powder with the mean particle size of approximately 2 mm. Then the powder was used for the obtaining of the tincture by double extraction with 70% ethyl alcohol. GW tincture is dark green liquid with a characteristic odor. For obtaining of the extract the powder was twice extracted with water at 90°C, filtered under vacuum and concentrated using a rotary evaporator. GW dry extract is a brown powder with a characteristic pleasant odor. The yield of extraction equaled 23%. The technology is standardized, corresponds to the requirements of State Pharmacopoeia of Ukraine, it was previously described in details [6,13,17].

Animal groups and treatment

The animals from the local colony of random-bred albino Wistar rats of the Central Scientific-Research Laboratory of the National University of Pharmacy were used. Male rats with the initial body weight of 320 g to 360 g (approximately 12 months of age) were kept under controlled standard conditions, on a natural light-dark cycle. All the protocols were approved by the Bioethics Commission of the National University of Pharmacy and were in accordance with "Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes".

A modified model of lipid metabolism disorders developed by AI Gozhenko and SG Kotjuzhinskaya [18] was used, that presupposed administration of protamine sulphate two times a day (10 mg/kg per day intramuscularly, the possibility of using this route of administration is substantiated in [19]) against the background of atherogenic diet. The modification consisted in the addition of 20% animal fat to the diet composition [20] and the intragastric administration of cholesterol (oil solution) at a dose of 40 mg per animal once a day [21], within the 16 days term of the study.

The animals were divided into 7 groups, as follows:

- intact control (IC);
- dyslipidemia (untreated control, UntrC);
- dyslipidemia + 50 mg/kg imtragastrically;
- dyslipidemia + GW tincture, 1 ml/kg intragastrically;
- dyslipidemia + metformin, 50 mg/kg intragastrically + GW tincture, 1 ml/kg intragastrically;
- dyslipidemia + GW extract, 1 g/kg intragastrically;
- dyslipidemia + metformin, 50 mg/kg intragastrically + GW extract, 1 g/kg intragastrically (n=5-7 in each group).

Metformin was used at the dose of 50 mg/kg intragastrically as it is expedient to use respectively low doses while exploring the possibility of synergism (still this dose was found to be effective on the model of diabetes [22]).

The dose of GW tincture 1 ml/kg intragastrically was chosen since being safe in the intact animals [5,13] it has demonstrated efficacy in animals with carbohydrate and lipid metabolism disorders [6,9]. Ethyl alcohol was removed from the tincture ex tempore.

The dose of GW extract 1 g/kg intragastrically was chosen since it was effective in rats with the disorders of lipid metabolism [12] as well as on the models of the kidney injury [7,8,17]. The extract was administered in the form of aqueous solution prepared ex tempore.

The interval between the administration of GW preparations and metformin equaled 40 min to minimize interaction at the level of absorption. The rats of the IC and UntrC groups received drinking water by the similar scheme. A 0.9% solution of sodium chloride was also injected intramuscularly to the rats of IC group instead of protamine sulphate solution.

On day 15 min, 40 min after the drugs administration (protamine sulphate, but not cholesterol was also administered), the status of excretory renal function (ERF) was determined: after administration of water loading at a rate of 3% of body weight, urine was collected for two hours (the rats fasted for 12 h before). The animals were previously adapted to the conditions of the experiment. Heparinized blood samples were drawn by exsanguination from barbiturate-anesthetized animals and plasma was separated immediately by centrifugation (the anticoagulant heparin *in vitro*).

The generally accepted routine biochemical methods were applied for blood plasma and urine analysis. Sodium and potassium content in urine, sodium concentration in blood plasma was measured using flame photometry method, creatinine – by Jaffe reaction, urea – by the reaction with diacetyl monooxime. Protein concentration in urine was assayed by reaction with sulphosalicylic acid. Plasma total protein concentration was measured by biuret method, albumin level – by the bromcresol green procedure. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity in plasma were determined according to the method of Reitman and Frankel and De Ritis ratio was calculated. Alkaline phosphatase (ALP) activity was assayed by measurement of the amount of phenol liberated from the hydrolyzed substrate (this value was measured also in urine), gamma-glutamyltransferase (γ-GT) activity – by the kinetic method using γ-L-glutamyl-3-carboxy-4-nitroanilide as a substrate and glycylglycine as an acceptor. Commercially-available kits from Filisit-Diagnostika (Ukraine) and Erba Lachema s.r.o. (Czech Republic) were used.

By the generally accepted formula, creatinine, protein, sodium and potassium excretion were calculated, as well as urine Na^+/K^+ ratio. Creatinine and sodium concentration in plasma and urine were also used for the estimation of glomerular filtration rate (GFR), relative water reabsorption (RH_2O), absolute and relative sodium reabsorption, its proximal and distal transport ($RpNa^+$ and $RdNa^+$) [23].

Medians, 25% and 75% percentiles (upper and lower quartiles) were calculated as well as the traditionally used arithmetic means and their standard errors (M ± m). Taking into account a problematical character of multiple comparisons in pharmacology and toxicology [24], the comparison of the central tendencies of independent samples was performed by the nonparametric Mann-Whitney U test. The level of significance was defined as $p<0.05$. Statistica 6.0 (StatSoft, Inc.) was used for the analysis.

Results and Discussion

It has been shown that the disorders of the lipid and carbohydrate metabolism as well as the mild changes of the histological structure of the internal organs are evident in the rats within the terms of our experiments [14,15]. Studies of the kidney function have revealed that the atherogenic diet combined with protamine sulphate administration even after the respectively short term led to the significant decline in GFR with the consequent decrease in diuresis, while water reabsorption was not changed (Table 1). Thus, the regulatory reaction typical under the conditions of water diuresis - an increase in GFR with water reabsorption decline, was not realized in rats of the UntrC group. Plasma sodium also have not undergone any shifts (Table 2), still the filtered load of sodium was reduced almost twofold because of GFR decline, the proximal and the distal transport were respectively decreased. A stable tendency to the enhancement in the relative reabsorption of sodium was seen ($p=0.088$ *vs*. the value of the IC group) and in the most of the rats of this group this value reached 99.98% to 99.99%, thus, a significant fall in urinary sodium excretion was observed (Table 1). Potassium excretion was decreased to a lesser degree and urine Na^+/K^+ ratio was reduced.

Hyperlipidemia, together with the significant disturbance of the liver lipid composition, was manifested in the studied animals as described by Tovchiga et al. [14] and it is known that renal injury is developed against the background of the disorders of the lipid metabolism and obesity. The latter was not seen within the term of the study as the body weight only tended to the increase in all of the groups receiving the atherogenic diet combined with protamine sulphate, except for the group receiving the extract per se (in which this increase was limited, data not shown). Still, among the early functional effects of the metabolic disorders linked to obesity on the kidney, there is an increase in tubular sodium reabsorption and an impairment of pressure natriuresis [25,26] that is consistent with our results. Intravascular volume expansion (due to the activation of the pressor systems in obesity [25,26]) might be enhanced by the well-known vasodilator effect of protamine sulphate which releases NO from systemic arteries, interferes with the mechanisms

	Intact control	**Dyslipidemia (untreated control)**	**Dyslipidemia + metformin, 50 mg/kg**	**Dyslipidemia + *A. podagraria* extract, 1 g/kg**	**Dyslipidemia + metformin, 50 mg/kg + *A. podagraria* extract, 1 g/kg**	**Dyslipidemia + *A. podagraria* tincture, 1 ml/kg**	**Dyslipidemia + metformin, 50 mg/kg + *A. podagraria* tincture, 1 ml/kg**
1	2	3	4	5	6	7	8
Diuresis, ml/ 100 g for 2 h	2.37 ± 0.19 **2.24** (2.14-2.53)	1.64 ± 0.24 **1.42** * (1.39-1.96)	1.02 ± 0.12 **1.12** *** # (0.88-1.18)	1.30 ± 0.14 **1.19** *** (1.03-1.53)	1.12 ± 0.30 **0.85** * (0.63-1.28)	1.71 ± 0.20 **1.87** * $$ (1.36-1.91)	1.24 ± 0.13 **1.27** *** (0.95-1.51)
GFR, ml/min for 100g	0.409 ± 0.062 **0.409** (0.280-0.532)	0.254 ± 0.030 **0.240** * (0.220-0.276)	0.269 ± 0.044 **0.239** (0.197-0.312)	0.411 ± 0.059 **0.374** (0.281-0.562)	0.370 ± 0.074 **0.275** (0.256-0.430)	0.373 ± 0.049 **0.361** (0.296-0.470)	0.221 ± 0.024 **0.203** * ^^ @ (0.185-0.265)
Relative R of H_2O, %	94.83 ± 0.63 **93.97** (93.89-96.04)	95.13 ± 0.58 **94.92** (94.14-96.30)	96.38 ± 0.60 **96.80** (95.04-97.82)	97.16 ± 0.32 **97.35** ** # (96.82-97.43)	97.33 ± 0.59 **97.83** * # (96.16-98.29)	95.84 ± 0.60 **95.97** (94.84-96.83)	94.99 ± 0.82 **95.79** ^^ (93.86-95.88)
Filtered load of Na^+, µM/min per100 g	72.0 ± 10.2 **74.5** (64.8-81.7)	39.5 ± 4.75 **35.5** * (31.8-50.0)	41.4 ± 7.13 **34.5** * (29.4-49.0)	61.9 ± 8.49 **62.1** #$ (41.9-81.2)	55.3 ± 12.3 **40.8** (37.1-64.2)	58.4 ± 9.35 **54.1** (44.3-76.9)	33.0 ± 3.88 **29.3** **^@ (27.3-39.3)
Relative R of Na^+, %	99.51 ± 0.04 **99.52** (99.46-99.57)	99.87 ± 0.10 **99.98** (99.92-99.99)	99.92 ± 0.02 **99.94** *** (99.92-99.95)	99.76 ± 0.05 **99.79** *$$ (99.71-99.84)	99.66 ± 0.07 **99.66**$$$ (99.55-99.79)	99.63 ± 0.10 **99.62**$ (99.41-99.80)	99.85 ± 0.11 **99.95**^ (99.92-99.98)
Rp of Na^+, mM /2 h per100 g	8.24 ± 1.22 **8.50** (7.30-9.44)	4.51 ± 0.54 **4.00** * (3.63-5.63)	4.82 ± 0.85 **4.03** * (3.31-5.73)	7.23 ± 1.01 **7.25** # (4.87-9.49)	6.47 ± 1.46 **4.79** (4.29-7.40)	6.74 ± 1.11 **6.27** (5.05-8.99)	3.77 ± 0.46 **3.32** *^@ (3.14-4.55)
Rd of Na^+, µM /2 h per100 g	361 ± 39.9 **327** (321-366)	226 ± 38.8 **204** (184-258)	153 ± 17.2 **158** *** (129-191	180 ± 19.1 **171** *** (147-208)	146 ± 40.6 **121** * (75.9-169)	247 ± 36.9 **235** $ (204-267)	182 ± 20.5 **179** * (136-223)
Sodium excretion, µmol/100 g for 2 h	43.0 ± 7.21 **41.3** (40.7-44.3)	6.86 ± 4.14 **1.03** *** (0.63-7.80)	3.52 ± 0.89 **3.22** *** (1.56-4.96)	15.6 ± 2.38 **13.7** ** #$$$ (11.0-20.7)	20.2 ± 4.63 **16.0** **#$$$& (15.0-23.2)	19.3 ± 3.94 **19.2** **#$$$ (13.7-26.7)	5.50 ± 3.96 **2.00** **^@ (0.92-2.45)
Potassium excretion, µmol/100 g for 2 h	46.7 ± 5.05 **44.0** (43.1-45.7)	14.3 ± 2.98 **12.1** *** (10.8-14.2)	14.2 ± 3.43 **11.3** *** (8.06-16.2)	65.1 ± 8.11 **62.5** ###$$$ (45.2-84.4)	66.4 ± 12.7 **58.3** ###$$$&&&@ (43.4-88.1)	34.4 ± 7.77 **26.9** #$^ (17.1-45.4)	15.2 ± 1.85 **14.6** ***^^^ (11.6-19.1)
Urine Na^+ /K^+ ratio	0.926 ± 0.125 **0.958** (0.889-1.02)	0.144 ± 0.058 **0.079** *** (0.052-0.223)	0.273 ± 0.078 **0.192** *** (0.174-0.269)	0.250 ± 0.044 **0.210** *** (0.194-0.253)	0.305 ± 0.031 **0.342** *** (0.266-0.356)	0.995 ± 0.347 **0.535**# (0.225-1.83)	0.458 ± 0.353 **0.099** (0.060-0.214)

* - p < 0.05 compared to intact control; ** - p < 0.02 compared to intact control; *** - p < 0.01 compared to intact control; # - p < 0.05 compared to dyslipidemia (untreated control); ### - p < 0.01 compared to dyslipidemia (untreated control); $ - p < 0.05 compared to the group receiving metformin; $$ - p < 0.02 compared to the group receiving metformin; $$$ - p < 0.01 compared to the group receiving metformin; @ - p < 0.05 compared to the group receiving the tincture; ^^ - p < 0.02 compared to the group receiving the tincture; ^^^- p < 0.01 compared to the group receiving the tincture; & - p < 0.05 compared to the group receiving the tincture combined with metformin; &&& - p < 0.01 compared to the group receiving the tincture combined with metformin

Table 1: Influence of goutweed preparations and metformin on the kidney function and electrolyte homeostasis markers in dyslipidemic rats; Mean ± S.E.M; Q_{50} (Q_{25}-Q_{75}), n=5-8 in each group.

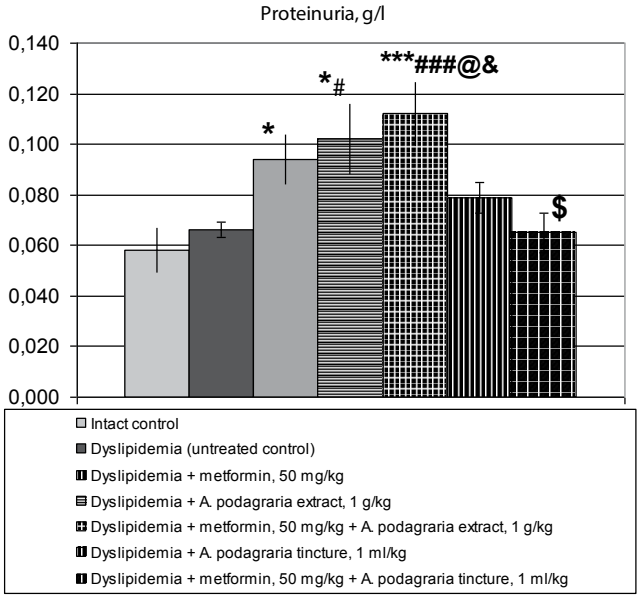
of calcium transport in vascular endothelium and causes reactions mediated by bradykinin and serotonin [27,28]. The indirect evidence of the constantly repeated hemodynamics shifts with the development of systemic hypotension and the respective cardiac responses was obtained in our previous studies in which the signs of myocardial dysfunction (granular dystrophy of cardiomyocytes, focal areas of the ischemic changes) developed despite the mild atherosclerotic changes in coronary vessels.

Under the conditions of the systemic vasodilation, the reactions of the kidney are directed to blood pressure maintenance through the enhancement of sodium and water reabsorption [29]. This may potentiate the increase in reabsorption caused by the metabolic shifts associated with dyslipidemia and obesity development which are discussed above. Within the terms of the study, the kidney functional changes have not yet resulted in the disorders of the histological structure. The changes in the tubules were limited to the granular dystrophy of epithelium allowing the maintenance of the intensive reabsorption processes [15]. The normal structure of glomerulus (assessed by light microscopy) was registered in all of the experimental groups

	Intact control	**Dyslipidemia (untreated control)**	**Dyslipidemia + metformin, 50 mg/kg**	**Dyslipidemia + *A. podagraria* extract, 1 g/kg**	**Dyslipidemia + metformin, 50 mg/kg + *A. podagraria* extract, 1 g/kg**	**Dyslipidemia + *A. podagraria* tincture, 1 ml/kg**	**Dyslipidemia + metformin, 50 mg/kg + *A. podagraria* tincture, 1 ml/kg**
Plasma creatinine, μM/l	75.8 ± 7.14 **72.6** (64.6-74.6)	76.7 ± 3.37 **76.9** (71.2-82.4)	66.8 ± 2.55 **65.8** # (65.0-70.9)	69.3 ± 3.94 **65.8** (65.8-67.9)	70.4 ± 7.57 **67.9** (58.4-79.8)	**72.4 ± 2.29** **70.2** (70.0-72.7)	79.9 ± 7.40 **79.3** (67.9-81.1)
Plasma sodium, mmol/l	159 ± 5.79 **161** (146-167)	159 ± 7.69 **160** (147-171)	150 ± 5.59 **148** (145-159)	150 ± 6.26 **146** (144-163)	160 ± 1.93 **160** (158-161)	151 ± 7.34 **153** (144-160)	146 ± 4.42 **149** (141-149)
γ-GT activity in plasma, μcat/l	0.033 ± 0.005 **0.031** (0.031-0.039)	0.024 ± 0.005 **0.022** (0.022-0.031)	0.043 ± 0.009 **0.041** (0.032-0.052)	0.029 ± 0.011 **0.031** (0.020-0.039)	0.034 ± 0.012 **0.035** (0.015-0.053)	0.028 ± 0.002 **0.028** (0.027-0.029)	0.033 ± 0.010 **0.022** (0.017-0.048)
Plasma urea, μM/l	5.64 ± 0.50 **5.77** (4.90-6.65)	3.66 ± 0.52 **3.10** * (2.68-4.71)	4.38 ± 0.34 **4.16** (3.71-4.95)	2.75 ± 0.36 **3.02** *** $ (2.46-3.31)	4.56 ± 0.35 **4.57** & ^^ (4.05-5.07)	3.35 ± 0.50 **3.55** * (2.81-4.09)	3.49 ± 0.31 **3.41** ** (2.89-3.72)
Urea excretion, mmol/100 g for 2 h	0.160 ± 0.023 **0.160** (0.129-0.185)	0.084 ± 0.017 **0.057** * (0.054-0.099)	0.062 ± 0.010 **0.057** *** (0.043-0.078)	0.089 ± 0.012 **0.088** * (0.074-0.101)	0.100 ± 0.027 **0.066** && (0.053-0.143)	0.085 ± 0.012 **0.084** ** (0.072-0.104)	**0**.041 ± 0.005 **0.039** *** ## ^^^ @@
Urea clearance, ml/min for 100g	0.255 ± 0.039 **0.281** (0.220-0.311)	0.214 ± 0.039 **0.219** (0.148-0.239)	0.118 ± 0.020 **0.106** ** # (0.078-0.171)	0.289 ± 0.053 **0.264** $$$ (0.209-0.309)	0.180 ± 0.053 **0.115** (0.101-0.226)	0.244 ± 0.052 **0.225**$$ (0.201-0.251)	0.093 ± 0.012 **0.101** ** ## ^^^ @ (0.071-0.118)

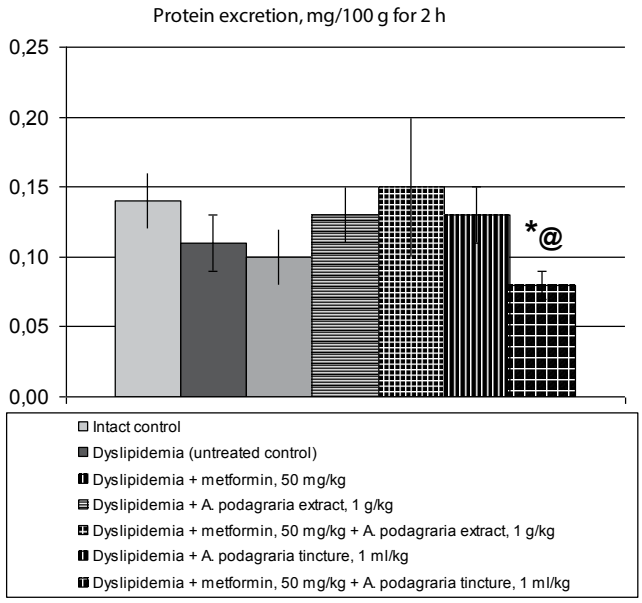
Table 2: Influence of goutweed preparations and metformin on the biochemical markers of plasma and the values of urea metabolism in dyslipidemic rats; Mean ± S.E.M; Q_{50} (Q_{25}-Q_{75}), n=5-8 in each group

* – p < 0.05 compared to intact control; ** – p < 0.02 compared to intact control; *** – p < 0.01 compared to intact control; # – p < 0.05 compared to dyslipidemia (untreated control); ## – p < 0.02 compared to dyslipidemia (untreated control); $ – p < 0.05 compared to the group receiving metformin; $$ – p < 0.02 compared to the group receiving metformin; $$$ – p < 0.01 compared to the group receiving metformin; & – p < 0.05 compared to the group receiving the tincture combined with metformin; && – p < 0.02 compared to the group receiving the tincture combined with metformin; ^^ – p < 0.02 compared to the group receiving the tincture; ^^^– p < 0.01 compared to the group receiving the tincture; @ – p < 0.05 compared to the group receiving the tincture; @@ – p < 0.02 compared to the group receiving the tincture



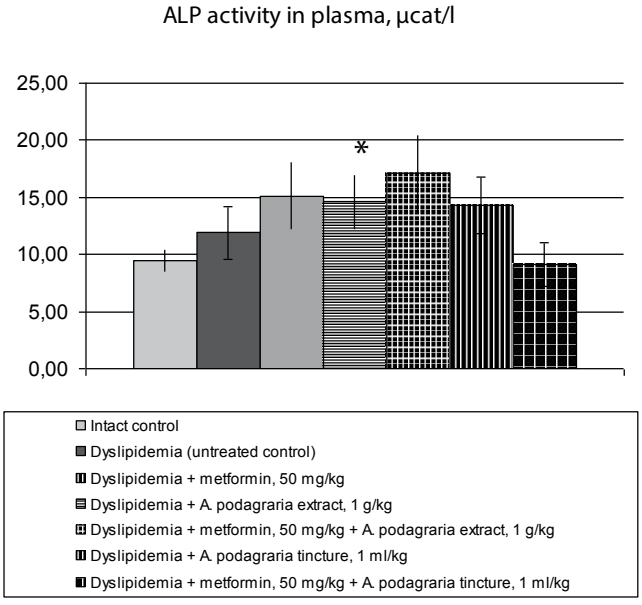
Values are expressed as Mean ± S.E.M; *-p<0.05 compared to intact control; ***-p<0.01 compared to intact control: #-p<0.05 compared to dyslipidemia (untreated control); ###-p<0.01 compared to dyslipidemia (untreated control); @-p<0.05 compared to the group receiving the tincture; $-p<0.05 compared to the group receiving metformin; &-p<0.05 compared to the group receiving the tincture combined with metformin.

Figure 1: Influence of goutweed preparations and metformin on proteinuria in dyslipidemic rats.



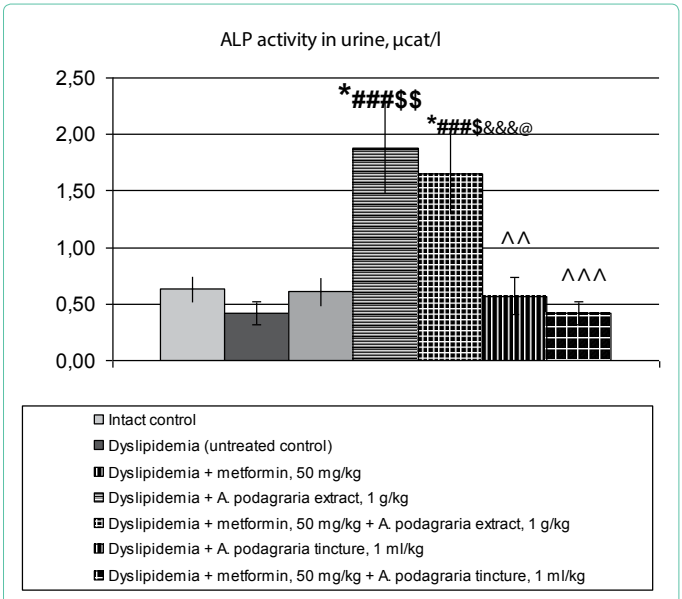
Values are expressed as Mean ± S.E.M; *-p<0.05 compared to intact control; @-p<0.05 compared to the group receiving the tincture.

Figure 2: Influence of goutweed preparations and metformin on protein excretion in dyslipidemic rats.



Values are expressed as Mean ± S.E.M; *-p<0.05 compared to intact control.

Figure 3: Influence of goutweed preparations and metformin on plasma ALP activity in dyslipidemic rats.



Values are expressed as Mean ± S.E.M; *-p<0.05 compared to intact control; ###-p<0.01 compared to dyslipidemia (untreated control); $-p<0.05 compared to the group receiving metformin; $$-p<0.02 compared to the group receiving metformin; &&&-p<0.01 compared to the group receiving the tincture combined with metformin. @-p<0.05 compared to the group receiving the tincture; ^^ p<0.02 compared to the group receiving the extract; ^^^ p<0.01 compared to the group receiving the extract.

Figure 4: Influence of goutweed preparations and metformin on ALP activity in urine in dyslipidemic rats.

receiving atherogenic diet and protamine sulphate as it corresponds well to the absence of the increase in the urinary protein level and ALP activity (Figures 1-4). These data also indicate the absence of the direct nephrotoxic effects of protamine sulphate at the used dose. Such effects might be expected proceeding from its significant accumulation in the kidneys of rats together with the slow excretion in the absence of exogenous heparin [30] and long known ability to interact with the negatively charged glomerular endothelial glycocalyx and to cause the retraction of podocyte foot processes leading to proteinuria [31,32]. Still *in vivo* proteinuria as well as the severe morphological changes was seen at doses higher than those used in our work [33]. Similarly, higher concentrations of protamine sulphate, than those that could be achieved after its administration to rats in our experiments, for its influence on paracellular conductance (with the neutralization of the negative charges at the luminal side) that has been widely used in the studies of the transport processes within the nephron (fixed entry charges theory of Lebedev [29,34] and other studies [35]). This influence (which could lead to the decrease in sodium reabsorption) was not evident and the described above obesity-induced increase in tubular sodium reabsorption is probable.

Decreased excretion can also be attributed partially to the reduced sodium intake because of the modification of the diet, the significant part of which (in contrast to the IC group) consisted of the animal fat, in which sodium content is low.

In the group receiving metformin plasma creatinine was significantly reduced (Table 2) that was not caused by the renal effects within the term of the study (extrarenal mechanisms

are possible). There were no other positive changes, diuresis was further reduced due to the increase in water reabsorption (p=0.08 *vs*. IC group value, Table 1), and there was a respective increment in urine protein concentration, while protein excretion and ALP activity in urine remained unchanged (Figure 1 and 2). The distal transport of sodium tended to decrease (p=0.09 *vs*. UntrC group value).

In rats receiving GW extract (per se or combined with metformin) water reabsorption was significantly enhanced (Table 1) with the simultaneous tendency to the increase in GFR (p=0.06 and p=0.11 *vs*. the value of the UntrC group, respectively) and without the significant changes in diuresis compared with the UntrC group. In the group treated with the extract per se the differences in GFR rate were also observed with metformin group (p=0.06). The ability of the extract at the studied dose to the interconnected influence on the main processes of urine formation with the increase in reabsorption has been proven previously both in the intact rats and on the different models of the kidney injury [8]. The increased GFR against the background of the extract per se (but not after co-administration with metformin) resulted in the significant normalization of the filtered load of sodium and its proximal transport. Nevertheless, the distal transport was not enhanced thus resulting in the decline in the relative reabsorption of sodium (p=0.06 and p=0.05 *vs*. the value of the UntrC group, in the groups receiving the extract and its combination with metformin, respectively) and its excretion. Potassium content in GW water extract is high and reaches 80 mg/g to 160 mg/g [7,10], and in the animals receiving this herbal preparation it is excreted sufficiently even under the conditions of kidney injury [8]. The same situation was observed in dyslipidemic rats (Table 1) and potassium was actively excreted in both groups receiving the extract. Urine Na^+/K^+ ratio remained decreased, since sodium excretion was enhanced to a lesser degree. In contrast to the previous data concerning the antiproteinuric effect of GW extract with the influence not only on protein excretion but on its concentration in urine (even if diuresis was decreased after polyuria overcoming [8]), in the dyslipidemic rats this effect was not evident and protein level was increased respectively to the reduction of diuresis. The same situation was observed in metformin group. ALP plasma activity tended to the increase in the most of the groups of animals receiving atherogenic diet and protamine sulphate (except for the rats receiving the tincture combined with metformin) and was significantly enhanced in animals receiving the extract, in this case the comparable increment of the enzyme activity in urine was also observed (Figure 3 and 4). Activity of γ-GT in blood plasma (Table 2) was not increased in all of the groups (including the rats treated with the extract) indicating the absence of severe cytolysis in the kidney and the liver (ALT and AST activity as well as De Ritis ratio also showed no difference between the groups except for the tendency to the decrease in ALT activity with the significant increment in De Ritis ratio in all of the groups receiving metformin, data not shown). The histological structure of the liver was not worsened in both groups receiving the extract compared with the other groups of treated animals [15] and there were differences in the structure of the kidney between the group treated with the extract per se and in combination, while the activity of ALP in urine was increased equally. Thus, the influence of the extract on ALP activity may be associated with metabolic changes rather than with cytolysis. Hypolipidemic drugs such as fenofibrate have been shown to increase the activity of this enzyme in hyperlipidemic animals [36] while PPARα activators are found among GW active substances, namely flavonoids [37]. Besides, the influence of GW components (especially, hydroxycinnamic acids) on the processes of bile formation or ALP functioning within the tubular epithelium is possible still we have not addressed these issues directly.

The increment of the enzyme activity in urine of the animals receiving GW extract could be attributed to the specificity of the used method, which was based on the measurement of the quantity of the liberated phenol after formation of its colored complex with 4-aminoantipyrine, in the presence of an oxidizing reagent. The metabolites of hydroxycinnamic acids containing phenolic hydroxyl groups could participate in this reaction, along with phenol formed from the added substrate. This is supported by the use of the similar reaction in the analysis of wine polyphenols [38]. Chlorogenic acid is one of the main active components in GW extract [7], and 3-hydroxyphenylpropionic acid has shown to be its major metabolite in rats [39]. Theoretically the levels of this compound recovered in rats urine [40] and the levels of plant phenolics reacting with 4-aminoantipyrine *in vitro* [38] are comparable (proceeding from the content of chlorogenic acid in the extract, which equaled 5% [9], the rats received in our study received 16 mg to 18 mg of this compound per animal given as a single dose as a solution, while in the work [40] the rats received 250 μM or 89 mg of chlorogenic acid per animal throughout the day with food). This value did not differ between the groups receiving the extract per se or combined with metformin (Figure 4) indirectly indirectly indicating that the latter did not influence on renal excretion of GW hydroxycinnamic acids metabolites.

GW tincture influence on the excretory renal function was manifested in the maintenance of sodium and potassium excretion with the normalization of urine Na^+/K^+ ratio. GFR, filtered load of sodium and its proximal transport approximated to the values of the intact animals, while the distal transport of sodium remained reduced. Thus, the relative reabsorption of sodium demonstrated a clear tendency to the decrease when compared with the UntrC group value (p=0.078). The increment of GFR (p=0,078 *vs*. the value of the UntrC group) with water reabsorption unchanged led to the relative increase in diuresis, which was the highest among the groups of the dyslipidemic rats. As in

the other groups discussed above, urine protein level (but not its excretion) was increased against the background of the reduced diuresis. The tincture per se did not influence on ALP activity in plasma and urine.

Combining metformin with *A. podagraria* L. tincture can be regarded as neutral for the kidney function as the most of the studied values did not differ from the group receiving monotherapy with metformin. The functional effects of the tincture were eliminated, still the influence on such important factor of the development of the kidney injury as proteinuria was achieved just in this group (in contrast with monotherapy). The combination as well as metformin per se has not change significantly the partial renal functions but prognostically unfavorable hyperfiltration has not developed. Minimal activity of ALP in plasma and urine (among the groups of the treated dyslipidemic rats) was registered in these animals. The previous data shown that the studied combination is able to influence positively on the other risk factors of the kidney injury: glucose metabolism disorders (complete normalization of glucose tolerance) and, partially, on the lipid metabolism disorders (normalization of the liver lipid composition) [14] and the beneficial influence of metformin on the kidney histological structure was also maintained in these animals [15]. In connection with these data and as described above, GFR decline within the term of the study is not a consequence of histological alterations but probably represents reversible functional changes. The results were obtained under the conditions of the forced diuresis and protamine administration while the additional studies using the spontaneous diuresis assessment might clarify the renal effects of the studied combination. The clinical relevance of the used model is also limited as an unphysiological constant excess of exogenous lipids was used. Under the conditions of a model with the primary insulin resistance (high dose of dexamethasone) the studied combination demonstrated the beneficial antiproteinuric effect (similarly to the current results) as well as neutrality in regards to the renal function with GFR and filtered load of sodium as well as its transport unchanged [41]. Besides, the low dose of metformin was used in our studies, while its efficacy on the models of diabetic nephropathy was established at a dose of 150 mg/kg and higher [42]. Previously the features of synergy antagonism were seen in the intact rats between metformin at low ineffective doses and low doses of the tincture [5]. The mechanisms of interaction of metformin with the herbal compounds are not studied enough. There are only data about the synergistic effect of the hydroxycinnamic acids and metformin on the glucose uptake by myotubes through the increase in GLUT4 expression [43] while the renal effects of such combinations are not described in the literature available. However, there are data that may explain the observed phenomena. Thus, activity of AMPK, which is one of the main targets of metformin, was shown to be reduced in kidneys of diabetic mice and the normalizing influence on AMPK/mTOR signaling axis contributes to the kidney protection in this case [44]. Different interactions are possible at the level of MPK signaling pathway, for example it has been shown that the compounds of herbal origin (including ferulic acid and quercetin, which are present in GW) do not induce a simultaneous AMPK activation and mTOR inhibition after associated administration in contrast with the individual use [45]. Similar interactions are possible for these compounds, which are present in GW, and metformin. Furthermore, AMPK is able to influence on numerous ion channels and transporters and regulate kidney tubular transport. AMPK is able to modulate functioning of Na^+/H^+ exchanger, epithelial Na^+ channel, Na^+–$2Cl^-$–K^+ cotransporter, ATP-dependent potassium channel, aquaporin-2 [46-48]. The changes of these systems functioning after coadministration of metformin and the studied herbal preparations are also possible and there are data of special interest concerning the regulatory role of AMPK for tubuloglomerular balance as well as tubular transport upon change of renal work load [49] and its activation by both a high- and a low-salt intake *in vivo* [50]. Still our assumptions are limited due to the complexity of the continuum that arose, namely the severe dyslipidemic conditions, protamine sulphate administration, low salt diet, water loading and the simultaneous use of metformin with the herbal drugs possessing intrinsic nephrotropic effects.

The functional changes in the animals receiving the extract combined with metformin may seem more beneficial than those of the combination with the tincture, still the influence on proteinuria is not achieved, activity of ALP in plasma and urine was increased. The previous data confirm the partial improvement of the lipid metabolism, but carbohydrate metabolism disorders were not corrected by this combination [14] and the normalizing influence on the histological structure of the internal organs was less pronounced than that of the tincture coadministered with metformin [15]. Lipid excess that occurs under the conditions of the model used forms the basis for an abrupt activation of lipid peroxidation processes and the phenolic compounds of the extract (which was used at a respectively high dose) may cause a multidirectional effects on lipid peroxidation/antioxidant system, so the prooxidative processes might be enhanced within the terms of the study.

Additional values of the protein metabolism were assessed, aiming to verify the safety of the studied drugs and reveal the possible renal effects. Plasma total protein and albumin level were not changed in all of the groups studied (data not shown, a slight albeit significant increase in total protein concentration was seen only in the group treated with the tincture combined with metformin). As to urea metabolism, the concentration of this metabolite in blood decreased in dyslipidemic rats, that may be the consequence reflect the suppression of its synthesis under the conditions of the lipids accumulation that is in complete accordance with

the data obtained in cafeteria-fed rats [51] (the decreased uptake of the amino acids by the liver was also proven in this work). Renal excretion of urea in the UntrC group decreased in concordance with the decline of its blood concentration and its renal clearance tended to the decrease (Table 2). In animals treated with metformin and its combination with the extract blood urea level was higher, its renal excretion was also reduced against the background of monotherapy and tended to the increase under the influence of the extract. The extract and the tincture per se did not influence on the urea level in plasma and its renal excretion compared with the UntrC group. Combining metformin with the tincture led to the further decrease in urea clearance that was not associated with the decline of GFR and diuresis.

The possibility of the decreasing of metformin active dose by combining it with *A. podagraria* L. tincture was established previously [6]. This study attempted to verify the possibility of supplementation of metformin pharmacodynamics with the additional favorable effects inherent in *A. podagraria* L. tincture and the extract. The features of synergy antagonism were seen between metformin and the constituents of the tincture, still the positive changes, among which the most important is antiproteinuric effect, were also revealed. The efficacy of metformin combination with the extract by a sum of criteria is lower, although the normalizing influence on the excretory renal function was seen.

Conclusions

- Atherogenic diet combined with protamine sulphate administration during 15 days results in the significant decline in GFR revealed in water loading test, decrease in filtered load of sodium, its proximal and distal transport as well as excretion. Diuresis, potassium excretion and urine Na^+/K^+ ratio are also reduced.
- Metformin (50 mg/kg) under the conditions of the study reduces creatininemia but leads to the further decrease in diuresis due to the enhancement in water reabsorption; proteinuria (but not protein excretion) is increased in these animals.
- Goutweed extract (1 g/kg), per se and combined with metformin (50 mg/kg) enhances water reabsorption with the simultaneous tendency to the increase in GFR and without the significant changes in diuresis, normalized the filtered load of sodium and its proximal transport, while the distal transport is not increased thus resulting in the higher sodium excretion. Potassium, which is present in the extract in high quantities, is excreted more actively and urine Na^+/K^+ ratio remains reduced.
- Goutweed tincture (1 ml/kg) normalizes sodium and potassium excretion as well as urine Na^+/K^+ ratio. GFR, filtered load of sodium and its proximal transport approximate to the values of the intact animals, water reabsorption is unchanged leading to the relative increase in diuresis, while the distal transport of sodium remains reduced.
- After combined use of goutweed tincture (1 ml/kg) with metformin (50 mg/kg) the renal effects of the latter, but not of the tincture, are realized. Antiproteinuric effect is achieved just in this group (in contrast with monotherapy) and minimal activity of ALP in plasma and urine (among the groups of the treated dyslipidemic rats) is registered.

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