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# Identification of natural products and their derivatives as promising inhibitors of protein glycation with non-toxic nature against mouse fibroblast 3T3 Cells

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## Abstract

This study was performed to identify new inhibitors of protein glycation *in vitro*. Protein glycation is one of the major causes of late diabetic complications. In this study, terpenoids and alkaloids, isolated from different medicinal plants, along with their derivatives, were evaluated for their antiglycation activity *in vitro,* while MTT assay on mouse fibroblast 3T3 cells was used to assess their potential cytotoxicity. Among the tested compounds, gossypol (2,2′-*bis*-(formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene) (1), isolated from *Gossypium herbaceum,* and its derivatives, gossypol acetic acid (2), gossypolidene- 4-aminoantipyrine (4), and gazolidone (6), showed a potent antiglycation activity ($IC_{50}$ < 16 $\mu$M), while gossypolidene-4-aminoantipyrine (5) showed a significant antiglycation activity with $IC_{50}$ value 82.934±2.924 $\mu$M, in BSA-fluorescence assay. Alkaloid, noscapine (3S)-6,7-Dimethoxy-3-[(5R)-4-methoxy-6-methyl-5,6,7,8-tetrahy-dro-1,3-dioxolo[4,5-g]isoquinolin-5-yl] isobenzofuran-1(3*H*)-one (7), isolated from *Papaver somniferum, N*-nitrosoaphyllinic acid (9), a derivative of alkaloid aphylline, and 2*H*-quinolizine, octahydro salt (11), a salt of alkaloid lupinine, exhibited significant inhibition activity with $IC_{50}$ values 152.662±5.432, 393.758 ±4.001 µM and 110.203±4.816µM, respectively. Similarly, compounds gossypolidene thiocarbamide (3), deoxypeganine hydrochloride (8), lupinine (10) and cytisine (12) showed moderate inhibition with $IC_{50}$ values of 401.865 ±18.450, 863.322 ±6.415, 712.176±7.745, and 728.462±2.331 µM, respectively. The results were compared with the standard antiglycation agent, rutin (13) ($IC_{50}$ = 98.012±2.030 µM).

Cellular cytotoxicity assay showed only gossypol acetic acid (2) and gossypolidene thiocarbamide (3) as somewhat toxic to 3T3 (mouse fibroblast) cells with $IC_{50}$ values 2.07 ±0.61 and 5.00 ±1.89 µM, respectively. Cycloheximide was used as a standard in this assay with $IC_{50}$ value 0.3 ± 0.089 µM.

**Keywords:** Protein glycation, diabetic complications, antiglycation agents, medicinal plants, cytotoxicity, terpenoids, alkaloids.

## Introduction

Non-enzymatic protein glycation and oxidative stress are closely associated with the development of diabetic complications [1]. Protein glycation is the coupling of free amino residues of protein with reducing sugars, such as glucose. This process is also known as Maillard reaction where amino groups of lysine, and the guanidine group of arginine often form adducts with the carbonyl moiety of reducing sugar [2]. The final products of these reactions are called advanced glycation end-products (AGEs). As a result of AGEs formation, protein structures and functions are altered [3]. The AGEs formation is thus strongly associated with diabetic complications. AGEs are complex organic products which cause damage to all vital organs, such as kidney (nephropathy), nerves (neuropathy), eyes (cataract), and blood vessels (atherosclerosis), and causes impaired wound healing as diabetes progresses [4-7].

The formation of AGEs gradually increases with normal aging, and age-dependent AGEs accumulate in human skin collagen, cartilage, and pericardial fluid [8]. Proteins with relatively longer half-lives, and with numerous lysine, hydroxylysine and arginine residues, including lens crystallins and collagens, are more vulnerable to accumulation of AGEs [9]. Thus protein glycation is a well known cause of normal, as well as pre-mature aging and other pathologies.

One of the key therapeutic approaches to prevent diabetic complications is inhibition of AGEs formation [10]. Thus, the discovery of AGEs inhibitors can contribute effectively in the prevention of diabetic or other pathogenic complications [11]. For

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example, a small synthetic hydrazine, aminoguanidine (AG), has shown promising inhibition of AGEs formation, and reached to phase III clinical trial. However, due to serious side effects, such as gastrointestinal problems, flu-like symptoms, and anemia, clinical trials were discontinued [12,13]. Rutin, a common flavonoid of fruits and vegetables, is a potent inhibitor of the AGEs formation *in vitro* and *in vivo* [14]. Rutin has also shown more inhibition of glycation of hemoglobin than the aminoguanidine [15-17].

Previously, efforts have been made to influence the process of protein glycation by preventing or slowing down the formation of AGEs [18]. However, so far no AGEs inhibitor has reached to clinical use due to one reason or the other. It is anticipated that in future, the antidiabetic therapy will involve the use of effective antiglycation agents to prevent associated complications.

In the present study, we evaluated several natural products and their derivatives for their antiglycation activity and associated cytotoxicity and some interesting results were obtained.

## Material and methods

### Chemicals

Bovine serum albumin (BSA) was purchased from Research Organics (USA), anhydrous D-glucose from Fisher Scientific (UK), rutin from Carl Roth GmbH & Co (Germany), and sodium azide and trichloro acetic acid (TCA) from Scharlau (Spain). Disodium hydrogen phosphate ($Na_2HPO_4$) and potassium chloride (KCl) were purchased from Merck (Germany). Sodium dihydrogen phosphate ($NaH_2PO_4$), potassium dihydrogen phosphate ($KH_2PO_4$), sodium chloride (NaCl), and sodium hydroxide (NaOH) were purchased from Sigma–Aldrich (USA). Sodium phosphate buffer 67 mM with pH 7.4 was prepared and sodium azide (3 mM) was added. Another buffer, called phosphate buffer saline (PBS) of pH 10 was also prepared.

Microtitre plate reader (SpectraMax M2 Microplate Reader, Molecular Devices, CA, USA) was used to measure the extent of protein glycation at 370 nm excitation and emission at 440 nm. Consumables purchased for cytotoxicity assay were mouse fibroblast (3T3) from the European American Culture Collection (EACC), MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) from Amresco (USA), Dulbecco's Modified Eagle's Medium (DMEM) and Fetal Bovine Serum (FBS) from GIBCO-BRL (USA), and penicillin and streptomycin from Sigma-Aldrich (USA).

### Bioassay Protocol for Antiglycation Activity In Vitro

A solution (10 mg/mL) of bovine serum albumin (BSA) was prepared in 67 mM phosphate buffer of pH 7.4, containing sodium azide (3 mM) to inhibit bacterial growth. A solution of anhydrous D-glucose (50 mg/mL) was also prepared in 67 mM phosphate buffer. Initially, unknown inhibitors (1 mM) were dissolved in DMSO, along with rutin as the standard inhibitor. In this assay, 96-well plate having 60 µL of the test sample in each well was used in triplicate. A blank sample containing only BSA dissolved in phosphate buffer, while positive control sample having BSA and glucose, were prepared and incubated for a week at 37 °C. To determine the inhibitory potential, 20 µL of unknown inhibitor was added into each well along with 20 µL of BSA and 20 µL of glucose. After incubation, 6 µL of 100 % TCA (trichloroacetic acid) was added to each well and the sample plate was centrifuged at 14,000 rpm for 4 minutes; pellets were formed at the bottom of the wells. 60 µL of PBS (pH 10) was added to dissolve the pellets for screening. Spectrofluorimeter was used to compare the fluorescence intensity at 370 nm excitation and emission at 440 nm [19-21]. Different concentrations of the potential inhibitors were used to calculate $IC_{50}$ values. EZ-fit Enzyme Kinetic Program (Perrella Scientific Inc., Amherst, U.S.A.) was used to calculate $IC_{50}$ values (µM). Rutin (1mM), exhibited 82.5% inhibition in this assay [16,22].

### Bioassay Protocol for Cytotoxicity

Cytotoxicity of terpenoids, alkaloids, and their derivatives were evaluated by using the standard MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay. For this purpose, 3T3 cells (mouse fibroblasts) were used. The same procedure was followed as reported in literature with slight modification. Cycloheximide ($IC_{50}$ = 0.3 ± 0.089 µM) was used as the standard [23,24].

## Results and discussion

Based on improved understanding of the adverse effects of the glycation process, it has become exceedingly important to manage this process efficiently, either by prevention of AGEs formation or by managing the consequences of their presence in body [25,26].

Discovery of promising antiglycation agents of natural and synthetic origins with high potency and low toxicity is urgently needed to control diabetic complications. Previously, terpenoids, alkaloid and their derivatives have shown inhibition against different biological disorders. Gossypol (1) is a polyphenol terpenoid, obtained from the seeds, roots, and stems of the cotton plant (*Gossypium herbaceum*). It is a crystalline yellowish powder having chemical and physical properties comparable to flavonoids. Previously, gossypol has shown significant medicinal potential, such as anticancer, antiviral, antibacterial, antitrypanosomal, and antimalarial activities [27-29]. Gossypol triacetic acid (2) is used to reduce body weight, intestinal length, and intestinal protein contents [30]. Since gossypol (1) has shown some serious side effects when used directly for contraceptive applications, efforts have been made to modify its structure to minimize toxicity and enhance therapeutic effects. A large number of gossypol derivatives, such as Schiff's bases, esters, and ethers have been prepared. Several gossypol derivatives and analogs, such as compounds 3- 6, possess activities such as antimalarial, anti-

cancer, HIV (human immunodeficiency virus), and antiparasitic properties [31- 37]. High throughput screening studies on a gossypol derivative 5 were performed *in vivo* and *in vitro.* It has shown significant anti-inflammatory and anthelmintic activities [38].

Noscapine (7), also known as (3S)-6,7-Dimethoxy-3-[(5R)-4-methoxy-6-methyl-5,6,7,8-tetrahy-dro-1,3-dioxolo[4,5-g]isoquinolin-5-yl]isobenzofuran-1(3*H*)-one, is a natural phthalideisoquinoline alkaloid. Previously, it has shown antitumor and cough suppressant properties [39]. Recently, it was isolated from dried capsules of *Papaver somniferum* L, which belongs to the family Papaveraceae [40]. Deoxypeganine is an alkaloid isolated from medicinal herb *Peganum harmala* L*,* which belongs to the family Zygophyllaceae*.* Deoxypeganine complex, deoxypeganine hydrochloride (8), is used in various medicines. It has major pharmacological potential against cardiovascular diseases, nervous system problems, gastrointestinal disorders, diabetes mellitus, and various tumors [41]. Aphyllinic acid, a synthetic derivative of alkaloid aphylline, obtained from *Anabasis aphylla L,* has shown branchoiospomodic and nerve-blocking activities. Several derivatives of aphyllinic acid were prepared, including nitrosoaphyllinic acid (9) [42].

Quinolizidines alkaloids possess a quinolizidine ring or a piperidine ring (such as 10, and 11). They are potential sources of medicines and have shown important pharmacological properties, such as antibacterial, antipyretic, antiviral, and hypoglycemic activities. An alkaloid (–)-lupinine, isolated from *Anabasis aphylla* L. has also exhibited ant cholinesterase activity [43-46]. Cytisine (12) is an alkaloid isolated from a plant *Thermopsis lanceolata* var. glabra (Czefranova)*. T. lanceolata* belongs to the family Fabaceae which is commonly found in the flora of Kazakhstan. Previously, cytisine has shown analeptic and anti-tobacco activities [47]

In the present study, several terpenenoids and alkaloids along with their synthetic derivatives were also evaluated for their protein glycation inhibitory potential and toxicity against 3T3 fibroblast cells *in vitro*. All tested compounds were purchased from Dr. Tlegenov R.T., Kara kalpak State University-742012, 5-1, Nukus city, Uzbekistan, and their identity and purity was re-checked in house.

Initially, all samples were evaluated for their inhibitory activity against protein glycation at 1 mM concentration. At this concentration, all compounds showed a significant inhibitory activity, especially compounds 1, 2, and 3 exhibited about 90% inhibition of protein glycation. The blank sample, which contained only BSA protein and glucose, showed 100% protein glycation in the absence of any inhibitor, as shown in Figure. 1.

The EZ-fit software (Perrella Scientific Inc., Amherst, U.S.A.) was used to calculate the $IC_{50}$ values (µg/mL). $IC_{50}$ values were measured by using different concentrations of the active samples

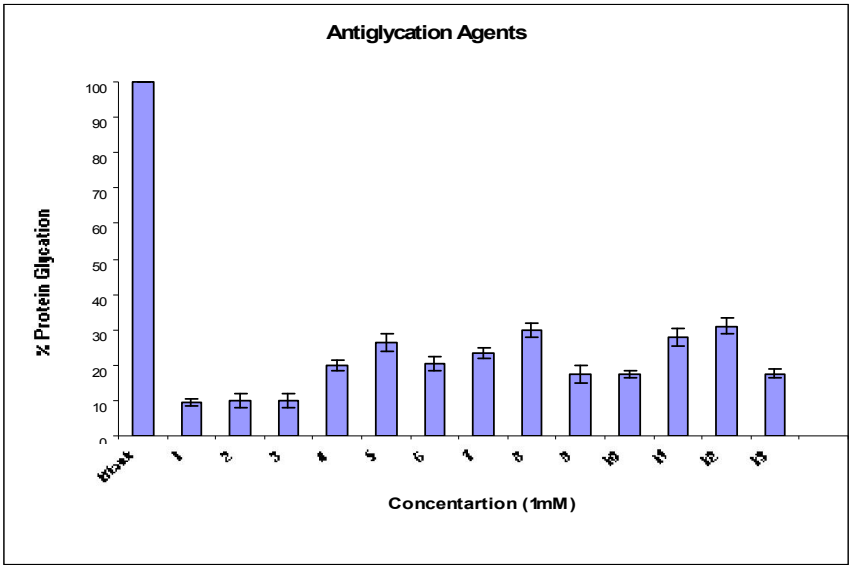


**Figure. 1.** Inhibition of protein glycation *in vitro*. The blank sample without any inhibitor showed 100% glycation.

**Table-1:** Antiglycation and cytotoxicity potential of natural compounds and their synthetic derivatives.

| Name | Source | Antiglycation ($IC_{50}$ ± SEM ) (µM) | Cytotoxicity ($IC_{50}$ ± SD) (µM) |
|---|---|---|---|
| Gossypol 2,2′-*Bis*-(formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene (**1**) | *Gossypium herbaceum* | < 16 | >100 |
| Gossypol acetic acid (**2**) | Gossypol (derivative) | < 16 | 2.07±o.61 |
| Gossypolidene thiocarbamide (**3**) | Gossypol (derivative) | 401.865 ±8.450 | 5.00±1.89 |
| 1-({[4-(4-Amino-3-methylphenyl)-2-methylphenyl]amino}methylene)-7-[8-7-oxo(2-naphthyl)]-3,8-dihydroxy-6-methyl-4-(methylethyl)naphtha len-2-one (**4**) | Gossypol (derivative) | < 16 | >100 |
| Gossypolidene-4-aminoantipyrine (**5**) | Gossypol derivative | 82.934 ±2.9246 | >100 |
| Gazolidone (**6**) | Gossypol derivative | < 16 | >100 |
| Noscapine, (3S)-6,7-Dimethoxy-3-[(5R)-4-methoxy-6-methyl-5,6,7,8-tetrahy-dro-1,3-dioxolo[4,5-g]isoquinolin-5-yl]isobenzofuran-1(3*H*)-one (**7**) | *Papaver somniferum L.* | 152.662 ±5.4326 | >100 |
| Deoxypeganine hydrochloride (**8**) | *Peganum harmala* (complex) | 863.322 ±6.415 | - |
| Nitrosoaphyllinic acid (**9**) | Aphylline (derivative) | 393.758 ±4.001 | >100 |
| Lupinine (**10**) | *Anabasis aphylla* | 712.176 ±7.7457 | >100 |
| 2*H*-Quinolizine, octahydro salt (**11**) | Lupinine (derivative) | 110.203 ±4.816 | >100 |
| Cytisine (**12**) | *Thermopsis lanceolata* | 728.462±2.00 | - |
| Rutin (**13**) | *Fagopyrum esculentum* | 98.012 ±2.030 | >100 |

All the tested compounds were then evaluated for the $IC_{50}$ values (µM) by using different concentrations. The results were expressed as mean ± SEM and the EZ-fit software (Perrella Scientific Inc., Amherst, U.S.A.) was used to calculate $IC_{50}$ values. Rutin (quercetin 3-rhamnosylglucoside) (13), a flavonol glycoside, was used as the standard inhibitor in this assay [48].

In this study, compounds 1, 2, 4, and 6 exhibited a potent antiglycation activity with $IC_{50}$ values less than 16 µM as compared to the standard antiglycation agent, rutin ($IC_{50}$ = 98.02±2.030 µM). Similarly, compounds 5, 7, and 11 also showed a significant antiglycation activity with $IC_{50}$ values 82.934 ±2.9246, 152.662 ±5.4326, and 110.203 ±4.816 µM, respectively. Compounds 3 and 12 showed a moderate inhibition with $IC_{50}$ values 401.865 ±8.450, and 728±2.00 µM, respectively, as shown in Table-1.

In general, the possible mechanism of action of alkaloids and its derivatives may involve interaction of N moiety of alkaloids with reducing sugar, thus making it unavailable to bind with the amino groups of proteins. Similarly, terpenes may show free radical scavenging activity and thus act as antioxidants to prevent the formation of advanced glycation end products.



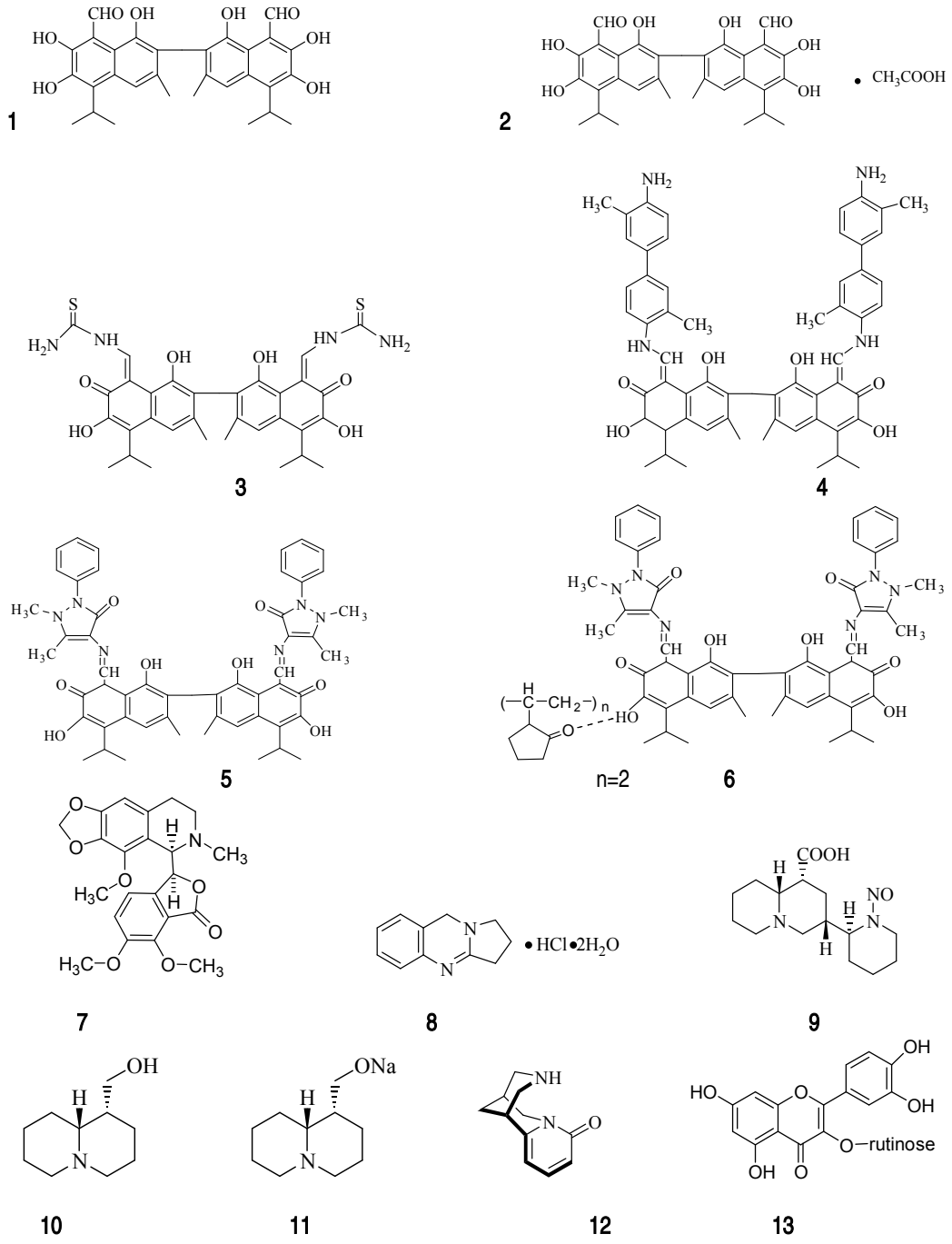


**Figure. 2.** Natural products and their derivatives evaluated for their anti-glycation activity *in vitro*.

In order to investigate non-toxic nature and safe use of these compounds, cytotoxicity studies were performed by using MTT assay on mouse fibroblast 3T3 cells. In this assay, only gossypol acetic acid (2) and gossypolidene thiocarbamide (3) were found to be toxic, with $IC_{50}$ values 2.07 ±0.61, and 5.00 ±1.89 μM, respectively. The cytotoxicity of compounds 8 and 12 could not be calculated due to unavailability of required amounts. All remaining samples were non-toxic, with $IC_{50}$ values more than 100 μM as compared to cycloheximide, which was used as the standard in this assay ($IC_{50}$ = 0.3 ± 0.089 μM).

## Conclusion

In conclusion, the results illustrate that most of the natural products, such as terpenoids, alkaloids, and their synthetic derivatives, have marked inhibitory activity against protein glycation without any cytotoxic effect. Furthermore, the present study opens the possibility of identifying safe and effective antiglycation agents for the management of late diabetic complications in future.

## Conflicts of interests

The authors declare no conflicts of interests.

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