

PREPARATION AND BIOLOGICAL ACTIVITY OF MOLECULAR COMPLEXES BASED ON SECONDARY METABOLITES OF *Glycyrrhiza glabra* L. AND *Sophora japonica* L.

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Abstract. Currently, nanopharmacology employs various approaches such as nanoparticles, nanocapsules, nanoemulsions and supramolecular complexes to improve drug solubility and bioavailability. Glycyrrhizic acid (GA), a natural triterpenoid from *Glycyrrhiza glabra* L., exhibits broad biological activities and due to its amphiphilic nature, can self-assemble into supramolecular structures. This property makes GA and its derivatives useful carriers for poorly soluble drugs. In this study, rutin, a flavonoid from *Sophora japonica* L., was selected to form complexes with GA and its monoammonium salt (MASGA) at molar ratios of 2:1, 4:1 and 9:1. The complexes were obtained by solvent evaporation and lyophilization and their structures were analyzed by UV and IR spectroscopy. Spectral shifts indicated hydrogen bonding and $\pi \rightarrow \pi^*$ interactions between rutin and GA/MASGA molecules. Biological evaluation in mice demonstrated that the complexes significantly enhanced capillary-protective activity compared with free rutin. Among the tested systems, the GA:Rutin complex at 4:1 ratio showed the highest activity, providing a three-fold reduction in vascular permeability at a dose of 5 mg/kg, while requiring twelve times less rutin than the free compound. These findings suggest that GA-based supramolecular complexes are effective carriers for rutin and the GA:Rutin (4:1) formulation represents a promising candidate for further preclinical studies.

Keywords: Metabolite, glycyrrhizin acid, rutin, supramolecular complex.

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1. Introduction

In today's modern world, numerous scientific and clinical studies are being conducted to evaluate the effectiveness and safety of medicinal plants in the treatment of serious diseases, including diabetes mellitus 2 type, atherosclerosis, hypertension, obesity, major depressive disorders, chronic infections and in general inflammatory processes caused by anthropogenic factors, among others. Glycyrrhizin acid (GA) is a remarkable natural triterpenoid compound extracted from the root of the licorice plant,

known for its wide range of biological activities, including anti-inflammatory, anticancer, antiviral and hepatoprotective effects (Asl & Hosseinzadeh, 2008; Schröfelbauer *et al.*, 2009; Zhao *et al.*, 2021). From a chemical structural perspective, GA is considered an amphiphilic molecule with a chiral structure, consisting of a hydrophobic triterpenoid aglycone moiety known as 18 β -glycyrrhetic acid, fused to a hydrophilic diglucuronic acid unit. The intrinsic amphiphilicity and chirality of the GA molecule impart properties such as hydrophobic interactions between the triterpene fragments and the tendency of the glucuronic units to self-assemble in water, forming supramolecular nanofibrils through hydrogen bonding (Li *et al.*, 2022). Elongated, semi-flexible GA nanofibrils, with a width of approximately 2.5 nm and a periodicity of 9 nm, formed by twisting, can give rise to more complex structures, including supramolecular hydrogels with a spatial hydrogen-bonded fibril network (Saha *et al.*, 2015). Moreover, the presence of numerous functional groups, a rigid crystal lattice structure and a unique conformation on GA molecules facilitates the formation of multifunctional hydrogel materials, making them ideal candidates for creating a wide range of GA derivatives (Ma *et al.*, 2019a; Gao *et al.*, 2021; Zhao *et al.*, 2022; Liu *et al.*, 2023). Interestingly, some studies have shown that the combined gel-like and amphiphilic properties of GA fibril assemblies enable them to serve as basic building blocks for the creation of novel multiphase colloidal materials, such as gel emulsions (Wan *et al.*, 2017; Li *et al.*, 2021; Du *et al.*, 2023) and ultrastable foams (Wan *et al.*, 2018; Ma *et al.*, 2019b; Su *et al.*, 2021). Recently, GA supramolecular hydrogels with versatile functional attributes have opened up various possibilities in fields such as drug delivery systems (Liu *et al.*, 2023; Li *et al.*, 2023a), response to specific stimuli, additive manufacturing (Li *et al.*, 2023b), injection and thixotropic advantages, regeneration and wound healing and biosensors (Zhao *et al.*, 2022; Li *et al.*, 2023b), due to their high bioactivity, modular mechanical properties, self-healing and adhesion. Considering the broad range of potential applications, it is crucial to understand and control the rheological properties of GA supramolecular hydrogels. The rheological behavior of these gels plays a vital role in determining their processing and functional performance in practical applications. Therefore, modulating gel rheology will be essential for assessing the specific requirements in various fields (Li *et al.*, 2023b). To create supramolecular complexes with GA, we selected Rutin, Vitamine P (3,3',4',5,7-pentahydroxyflavone-3-rhamnoglucoside), a flavonol-type flavonoid widely distributed in plants (Hosseinzadeh & Nassiri-Asl, 2014). Rutin possesses antioxidant, anti-inflammatory, anti-allergic, cytoprotective, vasoprotective, anticarcinogenic, neuroprotective, cardioprotective, antibacterial, antiviral, antiprotozoal, antitumor, anti-hypertensive antiplatelet, antispasmodic and hypolipidemic, activities (Nautiyal *et al.*, 2025) that have led to its widespread use in medicine and food supplements. Vitamin P is mainly used in the treatment of increased capillary fragility and capillary bleeding (Azeem *et al.*, 2024). However, current scientific research focuses on elucidating the role of rutin in the treatment of various chronic diseases such as cancer, diabetes, hypertension and hypercholesterolemia, as well as its broad-spectrum pharmacological benefits (Sharma *et al.*, 2013). Rutin has also been proposed as a promising agent for the treatment of Alzheimer's disease due to its antioxidant, anti-inflammatory and β -amyloid oligomer-reducing activities (Xu *et al.*, 2014), possible to use against some insects (Togaev *et al.*, 2024) or as a pain-relieving agent (Forouzanfar *et al.*, 2025).

However, for rutin, as for other flavonoids and in general, polyphenols, poor solubility is shown, which determines low bioavailability. In addition to this, rutin is

metabolised to different compounds by the gut bacteria after consumption. Therefore, the nanotechnology has been implemented for the improvement of the bioavailability of rutin using novel drug-delivery carriers (Nautiyal *et al.*, 2025). Therefore, the aim of this article is to obtain supramolecular complexes of rutin flavonoid from *Sophora japonica* L. with the *Glycyrrhiza glabra* L. metabolite - glycyrrhizin acid and its monoammonium salt in various ratios, to analyze the complex formation using spectral methods, to determine some of their physicochemical and biological properties.

2. Experimental section

2.1. Isolation and purification of glycyrrhizin acid

Extraction of Rutin from *Sophora japonica* L. Rutin was isolated from the buds of *Sophora japonica* L. The dried buds of *Sophora japonica* were crushed and extracted with water in a 1:15 ratio for 3 hours at 100-120°C. The aqueous extract was then filtered and left in the refrigerator for 24 hours. The precipitated technical rutin was filtered, dried and recrystallized in 70% ethanol. The yield was (17 %).

2.2. Obtaining supramolecular complexes

Preparation of the GA with Rutin (2:1) Complex: 2 mmol (1.65 g) of GA was completely dissolved in 25 ml of ethanol. Then, 25 ml of distilled water and 1 mmol (0.61 g) of rutin were added and the mixture was stirred vigorously. The mixture was left to stir at room temperature for 5-6 hours. Ethyl alcohol was removed by rotary evaporation and the aqueous phase was lyophilized. The yield was 1.77 g (91%). Melting point: 160-163°C.

Using this method, complexes of GA and rutin in ratios of 4:1 and 9:1 were also obtained.

Preparation of the MASGA with Rutin (2:1) Complex: 2 mmol (1.68 g) of MASGA was completely dissolved in 50 ml of 50% ethanol. Then, 1 mmol (0.61 g) of rutin was added and the mixture was stirred vigorously at room temperature for 5-6 hours. The ethyl alcohol was removed by rotary evaporation and the aqueous phase was lyophilized. The yield was 1.86 g (94%). Melting point: 176-179°C. Using this method, complexes of MASGA and rutin in ratios of 4:1 and 9:1 were also obtained.

2.3. Screening of the capillary-protective activity of the obtained complexes

Animals. In total, 48 male CD-1 mice, weighing 15-18 g, were used in the experiment. Manipulations with laboratory animals were carried out on the basis of the protocol of the Ethics Committee on work with laboratory animals in vivo, according to the International Recommendations of the European Convention for the Protection of Vertebrate Animals Used in Experimental Research (European Directive 2010/63/EU on the protection of animals used for scientific purposes. September 22, 2010. Official Journal of the European Union, L 276/33-L276/79). The animals were kept in vivarium conditions at normal room temperature (22-23°C) with free access to water and standard food. The experiments were approved by the local Ethics Committee (protocol No.9/06.02.23). The animals were randomly divided into 6 groups of 6 animals each.

Testing of cutaneous capillary permeability in mice. Changes in vascular permeability after administration of the obtained complexes were assessed by the time it takes for trypan blue to release into the site of xylene-induced inflammatory focus. Aseptic

inflammation was induced in the hind paws of mice by applying 0.02 ml of xylene. Ten minutes before the xylene application, 0.3 ml of a 0.3% aqueous solution of trypan blue was injected intraperitoneally. Two hours before the administration of trypan blue, the obtained complexes were administered to mice intragastrically at the dose of 5 mg/kg (6 experimental groups). The group of positive control received rutin at the dose of 10 mg/kg, the group of negative control received an appropriate volume of distilled water (Mironov *et al.*, 2012; Rakhimova *et al.*, 2023). To assess capillary permeability, the time it took for intraperitoneally injected trypan blue to appear at the site of aseptic inflammation (macroscopically visible change in skin color) was recorded (in min). *Statistical analysis.* The data were analysed by Microsoft Excel and were presented as “mean±Standard error of mean” ($M \pm m$). Comparison of groups (parametric analysis) was performed using Student's t-test for unrelated populations at a significance level of differences of $p < 0.05$.

2.4. Molecular modeling methodologies

A gas-phase (in vacuum) molecular dynamics (MD) simulation was conducted to investigate the potential interaction between GA and Ru in a 2:1 ratio. This simulation was performed in a large box using the GROMACS 2023 package (Abraham *et al.*, 2015; Páll *et al.*, 2020), as outlined in the literature (Konermann *et al.*, 2018). The AMBER99SB-ILDN (Lindorff-Larsen *et al.*, 2010) force field was employed, utilizing AM1-BCC charges on the atoms. Topology files of GA and Ru were generated by ACPYPE: AnteChamber PYthon Parser interfacE v. 2023.10.27 (Saha *et al.*, 2015). Initially, a cubic box ($V = 16633.73 \text{ \AA}^3$) measuring $25.52 \times 25.52 \times 25.52 \text{ \AA}$ was created for the two GA molecules, which were positioned $38 \text{ \AA}$ apart. Subsequently, a molecule of Ru was added to the box, resulting in a distance of approximately $94 \text{ \AA}$ between the Ru and the GA molecules. DFT optimization of the complex obtained from MD simulation was performed using the B3LYP/6-31G method, including dispersion correction (D3BJ (Grimme *et al.*, 2011)), with the ORCA 6 program package (Neese, 2022).

3. Results and discussion

3.1. Obtaining and properties of starting substance

Using methods known in the literature (Kondratenko *et al.*, 2005; Stolyarova *et al.*, 2008) to obtain the starting materials, GA (94% purity according to the HPLC standard) and MASGA (93% purity according to the HPLC standard) with higher purity than technical GA were obtained. Their physicochemical properties were determined and identified by comparing them with data in the literature.

HPLC chromatography was performed on an Agilent Technologies 1260 (USA) chromatograph. Chromatographic analysis conditions: column - Poroshell 120 EC-C18, $2.7 \mu\text{m}$, $4.6 \times 100 \text{ mm}$; detector - diode array detector (UV detector may also be used); eluent - acetonitrile: 0.5% acetic acid (35:65, isocratic method); flow rate - 0.75 mL/min ; detection - 254 nm ; column loading - $10 \mu\text{L}$; thermostat temperature - 30°C ; analysis time - 15 min .

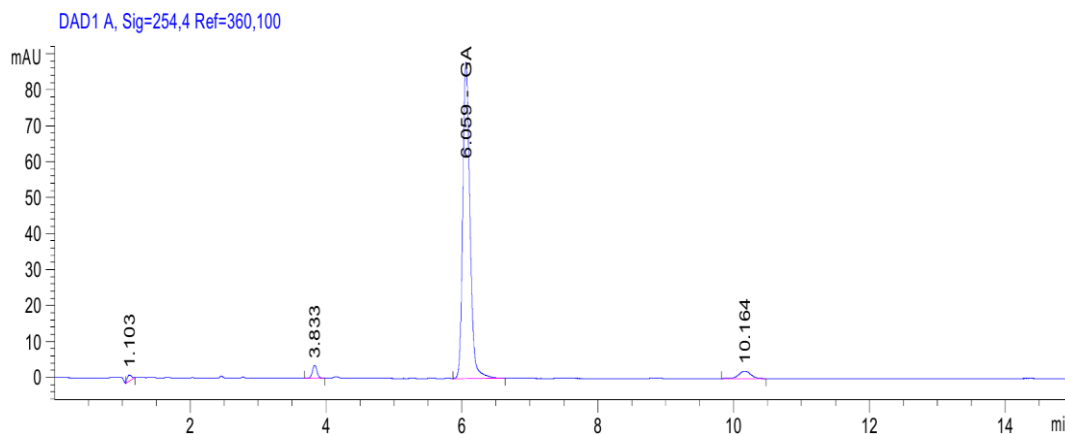


Figure 1. Chromatogram of GA in the HPLC

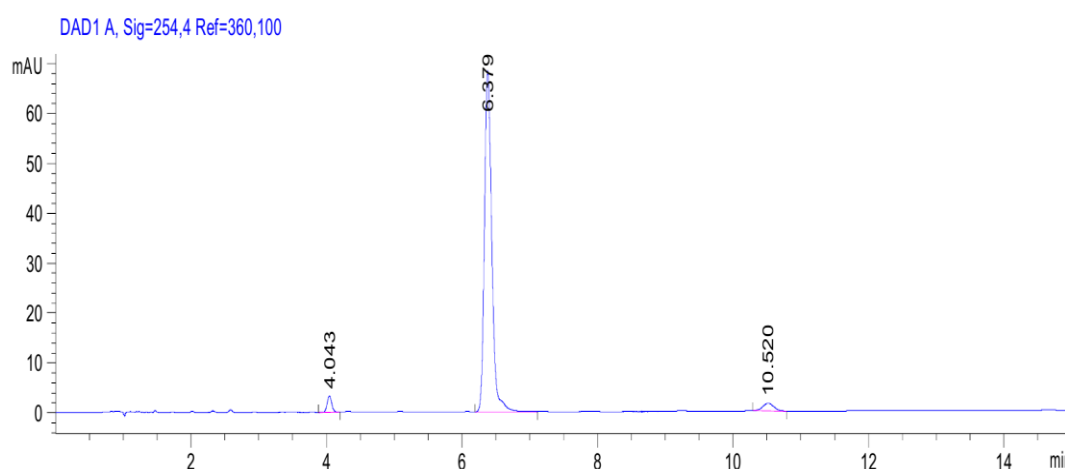


Figure 2. Chromatogram of MASGA in the HPLC

Rutin was isolated from the buds of Japanese safflower (*Sophora japonica* L.), which is grown as an ornamental tree. For this, the dried buds of the Japanese safflower plant were crushed and extracted with water for 3 hours at 100-120⁰C. The aqueous extract was filtered and left in the refrigerator for 24 hours. The precipitated technical rutin was filtered, dried and recrystallized from 70% ethanol. Rutin $R_f = 0.44$ (ethyl acetate: glacial acid: water 7.5:1.5:1.5). The purity of the obtained substances was determined by HPLC. The purity of rutin according to HPLC was 93% (Figure 3).

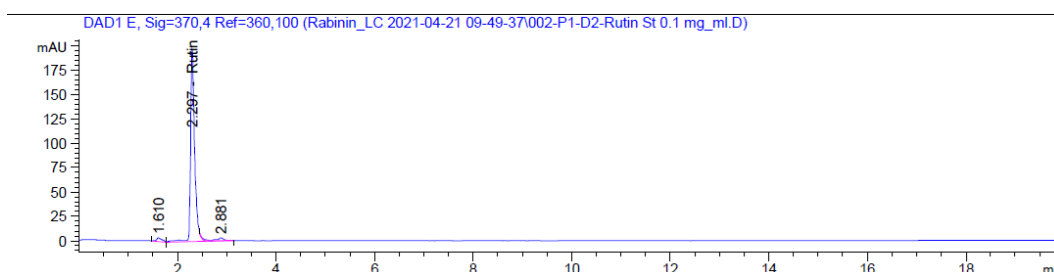


Figure 3. Chromatogram of Rutin in the HPLC

The following characteristic vibrational frequencies were determined in the IR spectrum of rutin: In the frequency range of 3600-3200 cm^{-1} , absorption bands corresponding to the stretching vibrations of all hydroxyl groups in the molecule are

observed. The vibrational frequencies in the region of $2950\text{--}2920\text{ cm}^{-1}$ in the spectrum are attributed to the methyl and methylene groups of rutin. Intense vibrational bands in the region of $1656\text{--}1599\text{ cm}^{-1}$ correspond to the carbonyl group. In the range of $700\text{--}900\text{ cm}^{-1}$, vibrational frequencies corresponding to the aromatic ring of rutin are observed.

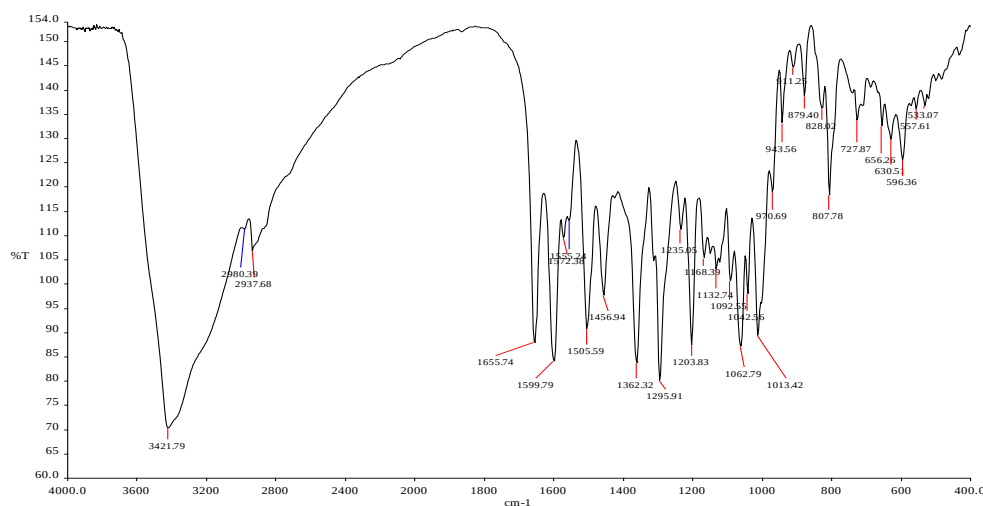
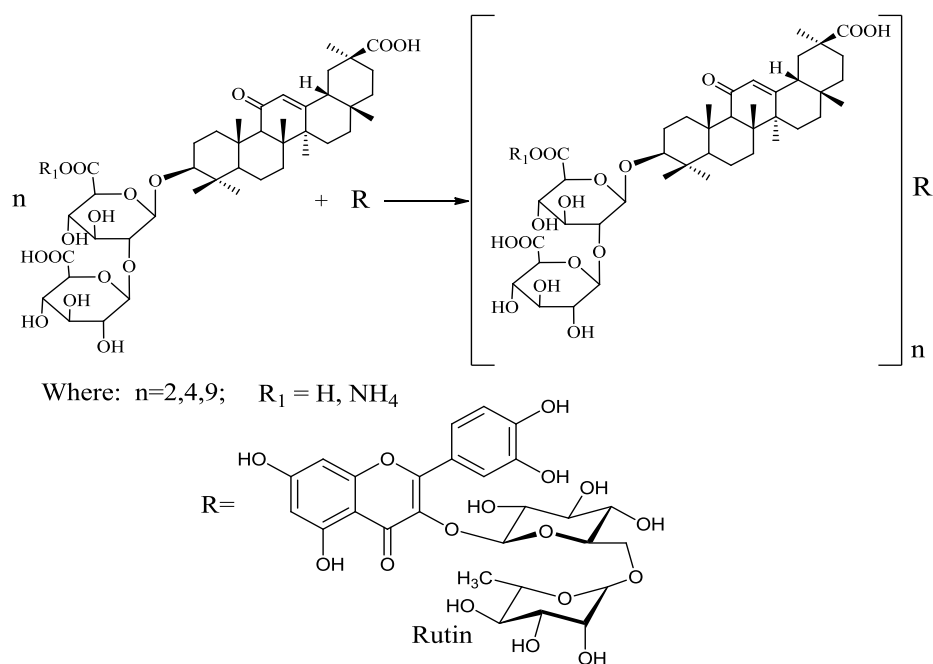


Figure 4. IR spectrum of rutin

3.2. Obtaining supramolecular complexes of rutin with GA and MASGA

Since GA is highly soluble in ethanol, it was dissolved in 96% ethanol. Then, water and rutin flavonoid were added with intensive mixing. MASGA, being well soluble in a water-ethanol system, allowed the complexes to be obtained directly in this medium. The complexing mixture was stirred for 5-6 hours. Ethyl alcohol was removed under vacuum and the aqueous portion was lyophilized. Using this method, complexes of GA and MASGA with rutin in ratios of 2:1, 4:1 and 9:1 were obtained (Scheme 1) and some physicochemical parameters were determined (Table 1).



Scheme 1. Preparation of Supramolecular Complexes of GA ($R_1 = \text{H}$) and MASGA ($R_1 = \text{NH}_4$) with Rutin (R)

An attempt was made to analyze the formation of complexes using UV and IR spectroscopic methods. Conclusions regarding the complex formation process were drawn based on the differences observed when the absorption maxima in the UV spectrum of the complexes were compared with those of the starting substances.

Table 1. Some physicochemical properties of GA and MASGA complexes with Rutin

Complexes	Mass ratio	Mass fraction of rutin in the complex	Melting point	Yield (%)
MASGA: Rutin	2:1	26,2%	170-172	92
MASGA: Rutin	4:1	15,1%	167-169	94
MASGA: Rutin	9:1	7,3%	165-167	93
GA: Rutin	2:1	27,1%	189-191	92
GA: Rutin	4:1	15,6%	187-190	91
GA: Rutin	9:1	7,6%	182-185	93

The UV and IR spectral data of all obtained complexes are given below:

Complex MASGA: Rutin 2:1. UV spectrum $\lambda_{\max}$, nm (lg ϵ) 255 (5.38), 361 (5.08). IR spectrum (ν , δ , cm^{-1}): $\nu(\text{OH})=3415$, $\nu(\text{CH}, \text{CH}_2, \text{CH}_3)=2944$, $\nu(\text{C=O})=1727$, $\nu(\text{C}_{11}=\text{O}, \text{C=C})=1656$, $\nu(\text{COO}^-)=1606$, $\delta(\text{CH}_2, \text{CH}_3)=1455$, $\delta(\text{CH})=1399$, 1365, 1307, 1207, 1172, $\delta(\text{C-O-C}, \text{C-OH})=1042$, $\delta(\text{=CH})=979$

Complex MASGA: Rutin 4:1. UV spectrum $\lambda_{\max}$, nm (lg ϵ) 252 (5.30), 363 (4.74). IR spectrum (ν , δ , cm^{-1}): $\nu(\text{OH})=3227$, $\nu(\text{CH}, \text{CH}_2, \text{CH}_3)=2946$, $\nu(\text{C=O})=1726$, $\nu(\text{C}_{11}=\text{O}, \text{C=C})=1656$, $\nu(\text{COO}^-)=1604$, $\delta(\text{CH}_2, \text{CH}_3)=1454$, $\delta(\text{CH})=1364$, 1307, 1211, 1172, $\delta(\text{C-O-C}, \text{C-OH})=1042$, $\delta(\text{=CH})=980$

Complex MASGA: Rutin 9:1. UV spectrum $\lambda_{\max}$, nm (lg ϵ) 251 (5.45), 362 (4.84). IR spectrum (ν , δ , cm^{-1}): $\nu(\text{OH})=3210$, $\nu(\text{CH}, \text{CH}_2, \text{CH}_3)=2978$, $\nu(\text{C=O})=1726$, $\nu(\text{C}_{11}=\text{O}, \text{C=C})=1658$, $\nu(\text{COO}^-)=1515$, $\delta(\text{CH}_2, \text{CH}_3)=1455$, $\delta(\text{CH})=1365$, 1264, 1214, 1172, $\delta(\text{C-O-C}, \text{C-OH})=1042$, $\delta(\text{=CH})=980$.

Complex GA: Rutin 2:1. UV spectrum $\lambda_{\max}$, nm (lg ϵ) 255 (5.11), 363 (4.81). IR spectrum (ν , δ , cm^{-1}): $\nu(\text{OH})=3380$, $\nu(\text{CH}, \text{CH}_2, \text{CH}_3)=2926$, $\nu(\text{C=O})=1731$, $\nu(\text{C}_{11}=\text{O}, \text{C=C})=1647$, $\nu(\text{COO}^-)=1516$, $\delta(\text{CH}_2, \text{CH}_3)=1455$, 1368, $\delta(\text{CH})=1319$, 1262, 1215, 1165, $\delta(\text{C-O-C}, \text{C-OH})=1043$, $\delta(\text{=CH})=982$.

Complex GA: Rutin 4:1. UV spectrum $\lambda_{\max}$, nm (lg ϵ) 253 (5.47), 363 (5.06). IR spectrum (ν , δ , cm^{-1}): $\nu(\text{OH})=3455$, $\nu(\text{CH}, \text{CH}_2, \text{CH}_3)=2949$, $\nu(\text{C=O})=1731$, $\nu(\text{C}_{11}=\text{O}, \text{C=C})=1650$, $\nu(\text{COO}^-)=1600$, $\delta(\text{CH}_2, \text{CH}_3)=1455$, 1389, $\delta(\text{CH})=1366$, 1320, 1262, 1215, 1167, $\delta(\text{C-O-C}, \text{C-OH})=1043$, $\delta(\text{=CH})=980$.

Complex GA: Rutin 9:1. UV spectrum $\lambda_{\max}$, nm (lg ϵ) 250 (5.32), 362 (4.69). IR spectrum (ν , δ , cm^{-1}): $\nu(\text{OH})=3349$, $\nu(\text{CH}, \text{CH}_2, \text{CH}_3)=2949$, $\nu(\text{C=O})=1730$, $\nu(\text{C}_{11}=\text{O}, \text{C=C})=1649$, $\nu(\text{COO}^-)=1516$, $\delta(\text{CH}_2, \text{CH}_3)=1455$, 1389, $\delta(\text{CH})=1365$, 1262, 1215, 1168, $\delta(\text{C-O-C}, \text{C-OH})=1055$, $\delta(\text{=CH})=982$

It was observed that the absorption maxima in the UV spectrum of the resulting complexes differed when compared to the spectra of the starting materials.

In the UV spectrum of MASGA, an absorption maximum was observed at a wavelength of 254 nm, corresponding to $n \rightarrow \pi^*$ electron transitions in the double bond conjugated to the carbonyl group located in the "C" ring of the MASGA molecule (Tolstikov *et al.*, 2007).

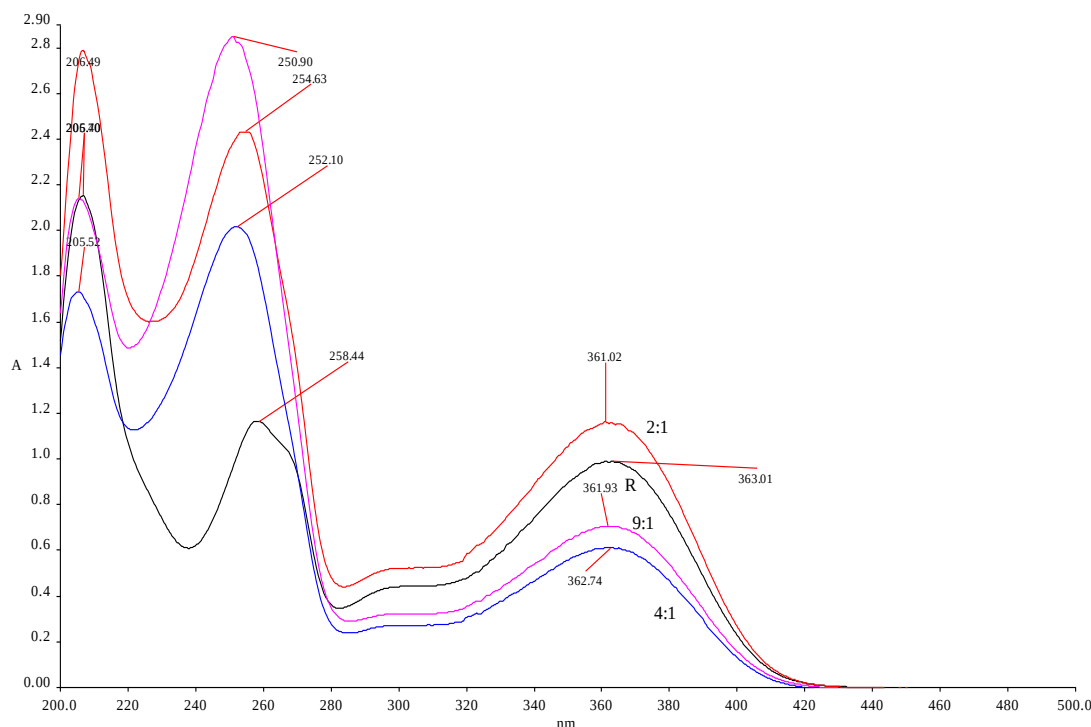


Figure 5. UV Spectrum of MASGA: Rutin Complexes

In the UV spectrum of rutin, the absorption maximum corresponding to the $\pi \rightarrow \pi^*$ transition appears at 258 nm, while an absorption maximum at 363 nm, resulting from $n \rightarrow \pi^*$ electron transitions, is observed with relatively low intensity (Nistor & Butnariu, 2023). The shift in these absorption maxima indicates that, in addition to the unshared electron pairs, the electron system in the π -bonds of the aromatic ring also participates in intermolecular interactions, with π bonds contributing to the formation of molecular complexes. From Figure 6, it can be seen that in the UV spectrum of the obtained supramolecular complexes, the absorption maxima corresponding to the $\pi \rightarrow \pi^*$ transition in the rutin spectrum are observed at the following wavelengths: 2:1 ratio (255 nm), 4:1 ratio (252 nm) and 9:1 ratio (251 nm). These shifts confirm the formation of supramolecular complexes based on the interaction between the triterpene molecule and the flavonoid. The following characteristic vibrational frequencies were observed in the IR spectrum of the MASGA: Rutin (2:1): In the wide, intense range of 3000-3600 cm^{-1} , the wavenumbers of the vibrational frequencies of all OH groups present in both starting materials were observed. In the region of 1726 cm^{-1} , the wavenumbers corresponding to the stretching vibration of the carbonyl group in the carboxyl groups of MASGA were observed. The vibrational frequency at 1657 cm^{-1} corresponds to the carbonyl group at C-11 in the MASGA molecule.

It should be noted that in the IR spectrum of the MASGA complex, the wavenumbers corresponding to the stretching vibrations of the OH group are observed at 3389 cm^{-1} . However, in the 2:1 MASGA complex, the wavenumbers corresponding to the stretching vibrations of the OH group shift to shorter wavelengths by 20-30 cm^{-1} . This phenomenon can be attributed to the formation of hydrogen bonds. However, the absence of such a shift in the MASGA: Rutin complex at a 9:1 ratio may be due to the sharp decrease in the amount of “guest” molecules available to form intermolecular hydrogen bonds.

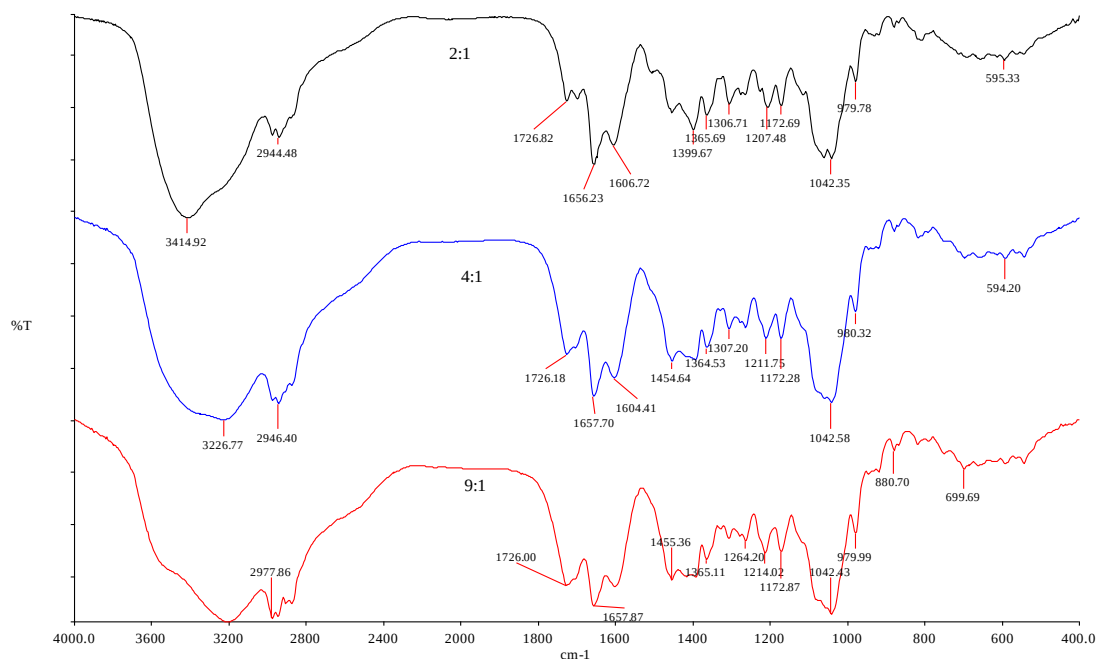


Figure 6. IR Spectrum of MASGA: Rutin complexes

3.3. Biological activity

The next step of studies is to determine how the process of complex formation influences the biological activity of complexes. Selon the Anatomical Therapeutic Chemical Classification System (WHO) for the classification of drugs and other medical products, rutin is determined as C05CA51 Rutoside, combinations: Bioflavonoids/Capillary stabilising agents/Agents for treatment of hemorrhoids and anal fissures for topical use/Vasoprotectives - Cardiovascular system. It has been used therapeutically to decrease capillary fragility. As shown rutin protects endothelial dysfunction through inhibiting Nox4 responsive oxidative stress, ROS-sensitive NLRP3 signaling pathway and substantial restoration of nitric oxide production (38-39). According with this the screening of biological activity of obtained complexes to choosing the perspective agent was provided using the simple pharmacologic test for assessment of capillary-protective properties on a large group of animals (Table 2).

Table 2. Capillary-protective activity of GA and MASGA complexes with rutin in Mice ($M \pm m$, $n = 6$)

Substances		Dose of complexes/ dose of rutin	Time, min.	Statistically significant differences, p			
				Control	Rutin	GA: Rutin, 4:1	MASGA: Rutin, 4:1
Control			5.80±0.5				
Rutin		10 / 10 mg/kg	8.80±0.9	p<0.05			
GA: Rutin	2:1	5 / 1.35 mg/kg	11.75±0.25	p<0.001	p<0.05	p<0.001	
	4:1	5 / 0.78 mg/kg	15.75±0.25	p<0.001	p<0.01		
	9:1	5 / 0.38 mg/kg	11.00±0.91	p<0.01		p<0.01	
MASGA: Rutin	2:1	5 / 1.31 mg/kg	9.75±0.25	p<0.01		p<0.001	p<0.01
	4:1	5 / 0.75 mg/kg	11.50±0.29	p<0.001	p<0.05	p<0.001	
	9:1	5 / 0.37 mg/kg	10.25±0.48	p<0.01		p<0.001	

In the control group, the time of trypan blue releasing at the site of xylene-induced inflammation was 5.80 ± 0.5 minutes. Rutin and all GA and MASGA complexes with rutin statistically decreased the capillary permeability: rutin - by 1.5 times, the complex GA: Rutin 4:1 - by 3 times and all other complexes - by 2 times. The capillary-protective activities of rutin and its complexes decreased in the following order: GA: Rutin (4:1) > GA: Rutin (2:1) > MASGA: Rutin (4:1) > GA: Rutin (9:1) > MASGA: Rutin (9:1) > MASGA: Rutin (2:1) > Rutin, with significantly higher capillary-protective activity of GA: Rutin (4:1) compared to the other complexes and rutin. Among the tested complexes of GA and MASGA in three molar ratios, the ratio 4:1 and GA as drug delivery system were found to be optimal. Since the structure of the complexes is supported mainly by hydrogen bonds, the presence of an additional free OH group in GA also had a positive effect on their biological effectiveness or the presence of the amino group in MASGA is the reason for the lower stability of the complex. It should also be noted that the dose of rutin is significantly reduced to achieve biological activity: when introducing complexes in a molar ratio of 4:1, the amount of rutin introduced is 12 times less and the effect is significantly higher. This proves the potential of using GC and MASGA as a safe carrier for poorly soluble substances with increased bioavailability, which in turn will reduce the doses of active components in the final product, limit side effects and increase economic profitability.

Thus, the 4:1 ratio of both GA: Rutin and MASGA: Rutin supramolecular complexes demonstrated high activity in both cases, with the 4:1 ratio of GA: Rutin showing significantly higher activity than the 4:1 ratio of MASGA:Rutin ($p < 0.001$).

3.4. Molecular modeling analysis

Firstly, molecular modeling of the Rutin with two molecules of GA has been performed by molecular dynamics simulations. Then, a complex obtained from MD simulation was studied by DFT method. MD simulation carried out in vacuum (in gas phase). Gas-phase simulations typically run without periodic boundary conditions (PBC), without Particle Mesh Ewald (PME) and without cutoffs for Coulomb or Lennard-Jones interactions. Therefore, it is proposed to use a large periodic box, ensuring that the periodic copies of the molecules are sufficiently distant to avoid interactions with the central box (Konermann *et al.*, 2018). And also, in vacuo MD simulations, the focus is solely on temperature-dependent properties and behaviors, not on pressure. In our case, the system, consisting of two molecules of GA and one molecule of rutin located in a cubic box, was minimized using the steepest descent algorithm with a convergence criterion of 100.0 kJ/mol·nm (Figure 7). Then, the equilibrium of the system in the NVT ensemble was carried out. For formal purposes, the equilibrium of pressure using the NPT ensemble was also carried out. As a result of the procedures, an interacting system was formed at the center of the cubic box.

After all procedures 200 ns production phase (MD simulation) using an integration time of 2 fs was performed. The RMSD graph has been built to estimate the localization of rutin and GA molecules during 200 ns MD simulation. According to RMSD graph the compounds quickly reached the equilibrium phase after an initial relaxation period. Starting from 10 ns, the system tends to stabilize, achieving stability during the simulation period (Figure 9).

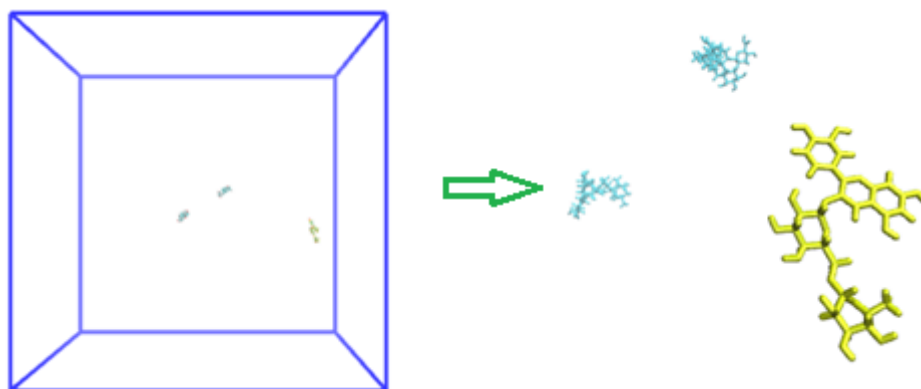


Figure 7. Separated localization of GA (cyan) and rutin (yellow) at the box

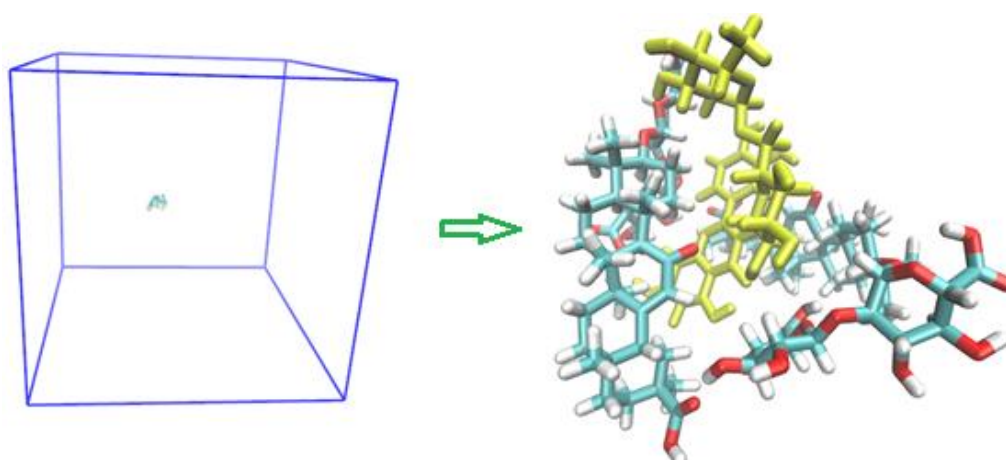


Figure 8. The localization of GA and rutin (yellow) at the box after NVT/NPT procedures

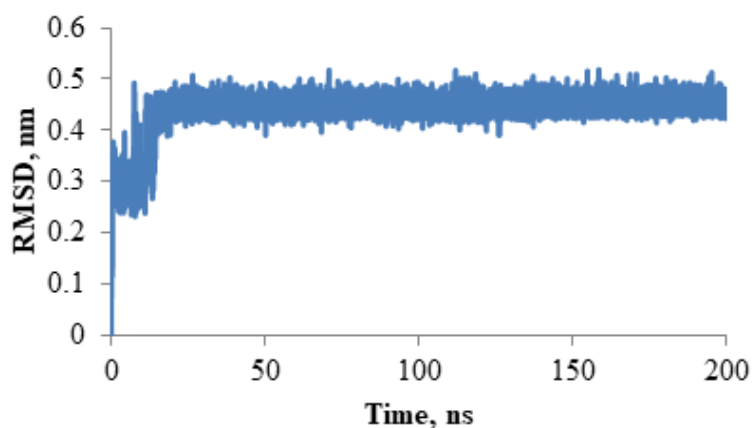


Figure 9. RMSD graph of the complexes as a function of time

When determining the optimal configuration of the molecular complex of glycyrrhizic acid (GA) and rutin (Ru) using molecular dynamics, it was found that the GA molecule, in a “head-to-tail” orientation, forms a dimer with a cyclic structure (Figure 4). In this structure, the carboxyl group of the aglycone part of the GA molecule forms a hydrogen bond with the OH group located at the C-3 atom of the second

glucuronic acid residue of the second GA molecule. At the same time, the OH group at the C-7 atom of the Ru molecule forms a hydrogen bond with the carboxyl group of the second glucuronic acid residue in the GA molecule. Moreover, hydrogen bonds are formed between the OH group at the C-3 atom of the second rutinose residue in the Ru molecule and the –O– atoms that connect the carboxyl and cyclic oxygen of the first glucuronic acid residue in the GA molecule, as well as link the glucuronic acid residues to the aglycone part. Additionally, the OH group at the C-3 atom in the Ru molecule forms a hydrogen bond with the carbonyl group of the aglycone part of GA.

Due to the aforementioned hydrogen interactions, the formed molecular complex can be considered sufficiently stable. The fact that the Ru molecule is situated between the cyclic dimer of GA and forms several hydrogen bonds with it provides even greater strength to the complex.

As a result of MD simulation the LJ-SR (Lennard-Jones) and Coul-SR (Coulombic) short-range interaction energies were calculated (Figure 8). The average Coul-SR and LJ-SR interaction energy for GA-Rutin showed to be $-150.82 \pm 5.3 \text{ kJ mol}^{-1}$ and $-204.33 \pm 0.18 \text{ kJ mol}^{-1}$, respectively. The complex obtained from molecular dynamics was further optimized using the DFT method (B3LYP-D3BJ/6-31G) with the ORCA 6.0 software package. During the DFT optimization, partial changes were observed in the hydrogen bonding system between the GA molecules. In particular, it was found that the carboxyl group of the aglycone part of the GA molecule forms hydrogen bonds with the OH groups at the C-3 and C-4 atoms of the second glucuronic acid residue in the second GA molecule, which is part of the “head-to-tail” cyclic dimer (Figure 11). At the same time, the hydrogen bonds formed by the Ru molecule were found to be identical to those observed during the molecular dynamics modeling stage. The interaction energy between the molecules of glycyrrhizic acid (GA) and rutin (Ru) in the framework of DFT calculations was determined using the formula: According to the calculation results, the interaction energy was found to be $E_{\text{int}} = -159.36 \text{ kJ/mol}$ (without considering the BSSE correction).

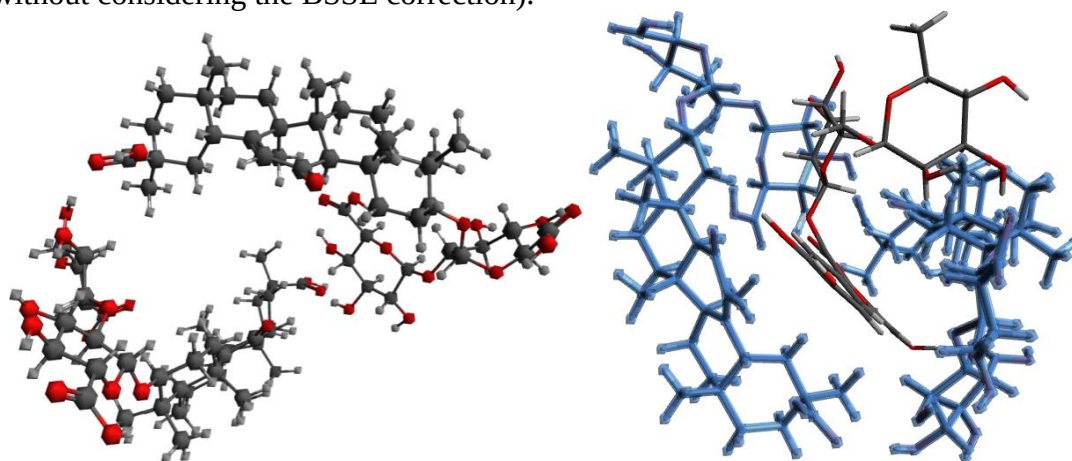


Figure 10. The complex obtained as a result of the MD simulation. In the left Rutin is hidden; in the right GA molecules is cyan colored

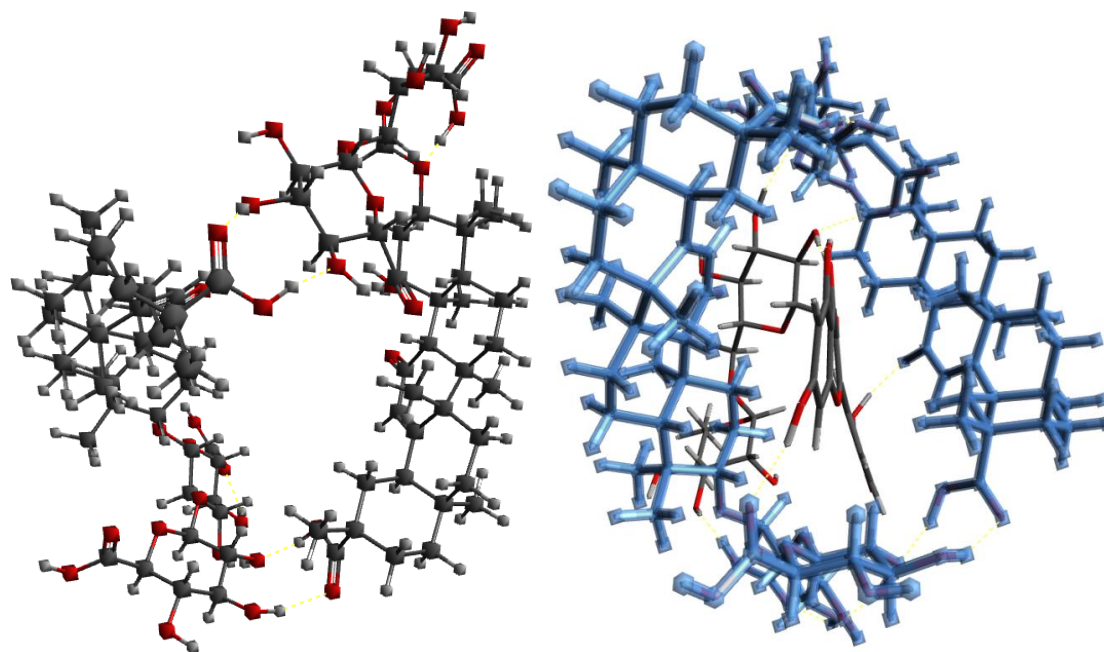


Figure 11. DFT optimized complex. In the left Rutin is hidden; in the right GA molecules is cyan colored

4. Limitations

This study has several limitations. First, the biological evaluation was restricted to a single model of capillary permeability in mice and additional analyses such as cytokine profiling, histopathology or broader pharmacological tests were not performed. Second, structural characterization of the complexes was based on UV and IR spectroscopy, supported by molecular modeling, but advanced techniques such as NMR, MS or XRD were not available to us. Third, the molecular dynamics simulations were conducted in the gas phase, which does not fully reproduce solvent or physiological environments. These limitations indicate that while the present findings strongly support the formation and activity of GA:rutin supramolecular complexes, further studies will be needed to validate their mechanisms and therapeutic potential under more diverse experimental conditions.

5. Conclusion

IR spectral analysis of the complexes revealed that the MASGA-rutin complexes exhibited shifts in the wavenumbers corresponding to the valence vibrations of the OH group. These shifts suggest that hydrogen bonds can form in the complex, such as (GA)-C=O...H-O-(Rutin) and -(H) O...H-O-. Moreover, as the amount of the “host” molecule increased in the complexes, bathochromic shifts of 4-7 nm in the absorption maxima were observed. These shifts, corresponding to $\pi \rightarrow \pi^*$ electronic transitions in the UV spectrum, indicate that the formation of the complex also involves electron participation from the p orbitals. Among the tested complexes of GA and MASGA in three molar ratios at the dose 5 mg/kg in terms of capillary-protective activity, the molar ratio 4:1 and GA as drug delivery system were found to be optimal and supramolecular Complex GA: Rutin (4:1) was recommended for further in-depth research as perspective agents.

Based on the calculations performed using Molecular Dynamics (MD) simulation and Density Functional Theory (DFT), it has been established that a stable molecular complex is formed upon interaction between GA and Ru. The MD simulation demonstrated that GA molecules form a cyclic dimer in a “head-to-tail” configuration, while the Ru molecule, situated within this structure, enhances the overall stability of the complex through the formation of several significant hydrogen bonds.

Structure optimization using DFT partially confirmed the results of the MD calculations and revealed some changes. In particular, the configuration of hydrogen bonds between GA molecules changed slightly and new interactions were formed; however, the hydrogen bonds between Ru and GA molecules were generally preserved. This indicates that the Ru molecule is stabilized within the complex.

The calculated interaction energy ($E_{\text{int}} = -159.36$ kJ/mol, without considering BSSE) indicates a high strength of the complex. This value confirms the presence of strong hydrogen and van der Waals interactions between GA and Ru molecules. Additionally, the Coul-SR and LJ-ST values obtained from the MD simulation also confirmed the effectiveness of these interactions.

Overall, the conducted research demonstrates that GA and Ru molecules can form pharmacologically significant complexes due to favorable intermolecular interactions. The amphiphilic properties of GA suggest its potential as a carrier for biologically active substances and a means to enhance their stability. It can be assumed that Ru exhibits more pronounced biological activity as part of the complex with GA, acting as an antioxidant flavonoid. The results of molecular modeling provide an important theoretical basis for further experimental studies and open up prospects for the pharmaceutical application of GA:Ru (2:1) complexes.

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