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Highlights

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PHARMA, DRUG DISCOVERY, TOXICOLOGY & MEDICINE

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## Szmodits László Pharmacist (Hungary. Budapest) Hungarian Pharmacist József Dorner, Pre-Eminent Botanist (1808-1873)

By Szmodits László

*Introduction-* The author describes the life and scientific work of the Hungarian pharmacist, József Dorner (1808-1873). Dorner worked for 16 years as a general pharmacist. But then turned to a special discipline of his profession and became an excellent botanist. He performed at first floristic research, but later concentrated his attention to plant anatomy and physiology, by systematic application of the microscope, he inaugurated a new aspect in botanica. Very soon, already in 1853, was Dorner discussing bioenergetics. He was selected to Corresponding Member of the Hungarian Academy of Sciences and was a distinguished botanical expert of the Royal Association of Natural Sciences. He has had been estimated as an excellent teacher as well.

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# Szmodits László Pharmacist (Hungary. Budapest) Hungarian Pharmacist József Dorner, Pre-Eminent Botanist (1808-1873)

Szmodits László

## I. INTRODUCTION

The author describes the life and scientific work of the Hungarian pharmacist, József Dorner (1808-1873). Dorner worked for 16 years as a general pharmacist. But then turned to a special discipline of his profession and became an excellent botanist. He performed at first floristic research, but later concentrated his attention to plant anatomy and physiology, by systematic application of the microscope, he inaugurated a new aspect in botanica. Very soon, already in 1853, was Dorner discussing bioenergetics. He was selected to Corresponding Member of the Hungarian Academy of Sciences and was a distinguished botanical expert of the Royal Association of Natural Sciences. He has had been estimated as an excellent teacher as well.

### *Life path until 1849*

József Dorner was of German nationality. Its original name was József Thurner junior wohl 1834. The time of the name change. Was confirmed by a letter from the Lutheran parish of Győr-Hungary. Was born in Győr on November 2, 1808 (1). His father József Thurner senior merchant in Győr. The mother Susanna Schmidt. He completed primary school at his birthplace and then an at Lutheran grammar school in Sopron. Here he liked botany, he studied the flora around Sopron with great interest (2, 3).

At that time was enough to complete 6 high school classes to enter the profession of pharmacist. In 1824, they practiced a Hungarian Crown Pharmacy in Sopron. Here he learned the Latin names of Medicines and took part in the formulations. Technologist also performed an operation (4). After 3 years, he passed the internship successfully. Between 1827 and 1831, he was an assistant in Pest and Bratislava (today Slovakia,) the old Hungarian name: Pozsony, German: Pressburg). After his time as an assistant, he enrolled at the University of Vienna, where he obtained a degree in pharmacy in 1832. In Vienna, he met the chief physician István Endlicher (1804.-1849), a botanist, who had a significant influence on the work of József Dorner.

In addition to German, he also spoke French. He was in contact not only with Hungarian botanists, but also with many foreign scientists. He became friendly with the famous botanist of those times: József Sadler (1792-1849) professors of botany at Pest university and János Heuffel physicians botanist and Antal Rochel (1770-1847) warden of the botanical garden in Pest and Ferenc Adoll Láng (1795-1863). pharmacist botanist. He kept up a correspondence with foreign scientists, including the botanists Eduard Fenzl (1808-1870) of Vienna and H. G. Ludwig Reichenbach (1793-1873) in Leipzig. His father bought the Golden Crown Pharmacy in Bratislava, in 1836. Here owned 4 floors. In 1840 he sold the Pharmacy.

Mediated by Antal Rochel in 1835, he visited of southern Hungary, Bács. He met János Heuffel, with whom they searched the local flora. He enriched his Herbarium with the plants collected there. His book on his experiences was published in 1839, Pressburg. „*Das Banat in toographisch-naturhistorischer Beziehung, mit besondener Berücksichtigung der Herculesbäder nächst Mehadia und ihrer Umgebungen.*” It was such a success that it aroused the interest of contemporary scientists. This has already been very much noticed in the country. In 1840, he sold his chemist's shop and took a post in the health department of the Governor's Council in Buda. (3,5).

He published two books on chemical technology: 1. on the theory and practice of vinegar production. 1841.- 2. the process of brandy with mashing, making malt and yeast, Pest, 1843. (1).

In 1842 the Hungarian Academy of Sciences announced a tender for the change of the climate of Hungary about, the flora and fauna- Dorner observed weather data every 24 hours, a day for 5 years in different directions. He also often recorded the temperatures of the water in a well in Buda, Tabán. He continued this work later until 1850. Was valuable because it dealt extensively with 100 gold honors by Academy in 1847 (5, 6, 7).

In 1846 he decided to compile and publish a manual on the flora of Hungary. Heuffel and Sadler welcomed Dorner's plan and offered to collaborate with him, but due to Sadler's death in 1848 and Heuffel's illness, the plan was never realized. Dorner could not undertake the enormous work alone (1, 4).

On March 13 the 1847, was elected a member of the Royal Hungarian Society of Natural Sciences. As one of the notabilities of the botanical committee. He was invited to take over the book collections of the Society. He organized the enlargement of the collections through exchange (8).

In 1848 during the war of independence he was invited to be a ministerial secretary to the Ministry of Religion and Public Education. However, after the capitulation of 1849. He returned to privacy. At that time he devoted all his to botany. (3, 9).

#### *His life and work from 1851 until his death*

In 1851-53 he gave several lectures in the Hungarian Society of Natural Sciences: parasitic plants, fertilization of plants and plant cells (10.11). In 1853 a book was published on grape diseases.

In 1853 he delivered a lecture at the Royal Hungarian Society of Natural Sciences with the title „The plant kingdom and man”. In a paper published in the journal „Új Magyar Múzeum” (New Hungarian Museum) he discussed the Darwinist theory of evolution:

*„The times when botany was confined to the identification, of genera and species... have passed. The new science has assumed a higher standing. It no longer satisfies itself with the classification of forms..., but searches for their inner relationships.”*

In the paper „The plant cell.”. He wrote: *„The study of cells in the most remarkable part of the science of botany, which with the introduction of the microscope has become a most interesting subject. There exists in the cell a remarkable moving force, so far physically unidentified, whereby the cell content spreads in different ways unnoticed in the body of the plant.”*

József Dorner realized that the tissue structure of plants would only become phenomenon of plants, which is based on motion and metabolism. At that time it was still novel in botany.

On December 15, 1858, he was elected a corresponding member of the Hungarian Academy of Sciences. The title of his inaugural lecture was: *„Online of the history and application of the microscope.”*

In the 1853, He taught natural science (biological subjects) in the Lutheran grammar school in Szarvas, Békés county. Then from 1860 in a similar school in Pest. Both schools taught in Hungarian, but he also taught Chemistry, Physics, German and French.

#### *Textbooks*

1. Elements of Botany, 1864. Pest,
2. Elements of Mineralogy: 1858, 1865, Pest,
3. Elements of Zoology:
  - Uninhabited: 1864, Pest,
  - Reptiles, fish, birds. 1863. Pest,
  - Mammals: 1863. 1874, Pest (2).

Dorner knows first-hand scientific achievements of the age. He advocated the need for permanent knowledge.

*„We don't want to train botanists and zoologists. We educate young people enriched with touring back ground, who are capable of self-education” (9).*

In 1855 published an article on the history of the fertilization of seed plants. He highlighted the role of seeds. Here (3).

In 1860 he wrote a very interesting article entitled „The Hungarian Great Plain, especially the area around Szarvas.” With a historical introduction, Hungary a country of peculiarities and extremes. At the time this settlement was a village.

Discussed in detail the natural village conditions (bedrock, climate, agricultural production) and the situation of the peasantry. Worked out the population density of the area. He also pointed out the benefits of urbanization. Perceived differences in social classes.

„He described the conditions of the poor and wasteful lives of the nobles (9).

In one of his last works, he compared the flora of Pest county that of Lower Austria. This study was completed with a detailed list of plants (1862).

In his paper „Oak-trees of Budapest”, published in 1862, he gave a summary of the Quercus species occurring in the environs of Budapest on the basis of his observations (3).

In 1863 at the IXth Congress of Hungarian Physicians and Natural Scientists, held in Pest, Dorner delivered a lecture on the „Cuscuta varieties of the Hungarian meadow.”

He pointed out that owing to their parasitic nature, the dodder, species differ from dicotyledons in their tissue structure. He presented details of the germination and development of the dodder, and mentioned the prevention of dodder infestation. During the lecture, he showed mature specimens of Cuscuta. This lecture was a great success and aroused the interest of many of foreign botanists as well (12).

Dorner taught the Hungarian botanical terminology to Paul Ascherson (1843-1913,) a botanist from Berlin, who then translated „Cuscuta varieties of Hungarian meadow” into German (5).

In 1868, in one of his last academic lectures, he came up with some rare plants from Hungary. Anthemidis neilreichii was also detected in Hungary, but was discovered in Lower Austria a few years ago. He also founded the species Cuscuta obtusiflora Hunp et Bonl, which had already been discovered by Paul Ascherson and Viktor Janka in the Lower Tisza (13).

He was constantly enriching his herbarium. The grammar school in Budapest kept its herbarium of 48 fascicles, 15 thousand plants. Today it is in the Budapest Gallery of the Natural History-Museum. He also wrote book reviews.

### József Dorner's human characteristics, disease, and death

He was a highly educated, and a very well-prepared humble man. He also acknowledged superstitious customs. Though his scholarly figure he can easily recognize superstars habits as well. He was always a success with the skill of an excellent performer. He was characterized by beautiful Hungarian speech. He justified what he had to say, He calced humor to his performances. He wo speak to hin listened intetly to hin.

He married in 1845, but had no children from marriage. His wife died early, so so he lived alone. He also taught it has long illness. He had an incurable heart problem. So died on 9 th October, 1873. Fiume Road in Budapest, his heas still rest in public cemetery today. The memory Hungarian Pharmacist Pantheon is preserved.

## II. SUMMARY

József Dorner was one of the first Hungarian researches in plant morphology and physiology with a degree in pharmacy. At that time, he was not yet able to teach at the university as an academy. But with a pen and word, he made great. He was an excellent instructor. I wrote in English so that, readers could get to know the work of Hungarian pharmacists.

*„Respect for the greats and noble tradition of the past, the main guarantee our ascension.”*

(Vilmos Milkó Hungarian doctor-professor, 1878-1956)

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# Pharmaco-Economic Evaluation of the Treatment used in Ophthalmological Practice

By Shoyusuf F. Shodmanov & Shakhnoza Z. Umarova

**Abstract-** The most important among the clinical forms of glaucoma is primary open-angle glaucoma (POAG), which, according to various authors, occurs in 70%-92% of cases. Due to the high prevalence of POAG, late detection and serious prognosis for visual functions, this disease occupies a special place in clinical ophthalmology. The aim of the study is to conduct a pharmacoeconomic evaluation of Tafluprostvs Travoprost in patients with POAG. Materials and methods of research is pharmacoeconomic methods of analysis, in particular the calculation of the relative risk, the calculation of the probability of events, cost-effectiveness analysis. According to the calculated results of pharmacoeconomic analyzes, Tafluprost was relatively less expensive and more clinically effective than Travoprost in patients with primary open-angle glaucoma. Alternative treatment with Tafluprost contributes to savings in the overall treatment procedure. Therefore, we recommend adding Tafluprost to the list of essential medicines.

**Keywords:** *pharmacoeconomic evaluation, ophthalmology, medicines, relative risk, probabilities of events, cost-effectiveness analysis.*

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PHARMACOECONOMIC EVALUATION OF THE TREATMENT USED IN OPHTHALMOLOGICAL PRACTICE

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# Pharmaco-Economic Evaluation of the Treatment used in Ophthalmological Practice

Shoyusuf F. Shodmanov<sup>α</sup> & Shakhnoza Z. Umarova<sup>ο</sup>

**Abstract-** The most important among the clinical forms of glaucoma is primary open-angle glaucoma (POAG), which, according to various authors, occurs in 70%-92% of cases. Due to the high prevalence of POAG, late detection and serious prognosis for visual functions, this disease occupies a special place in clinical ophthalmology. The aim of the study is to conduct a pharmacoeconomic evaluation of Tafluprostvs Travoprost in patients with POAG. Materials and methods of research is pharmacoeconomic methods of analysis, in particular the calculation of the relative risk, the calculation of the probability of events, cost-effectiveness analysis. According to the calculated results of pharmacoeconomic analyzes, Tafluprost was relatively less expensive and more clinically effective than Travoprost in patients with primary open-angle glaucoma. Alternative treatment with Tafluprost contributes to savings in the overall treatment procedure. Therefore, we recommend adding Tafluprost to the list of essential medicines.

**Keywords:** pharmacoeconomic evaluation, ophthalmology, medicines, relative risk, probabilities of events, cost-effectiveness analysis.

## I. INTRODUCTION

The most important among the clinical forms of glaucoma is primary open-angle glaucoma (POAG), which, according to various authors, occurs in 70%-92% of cases. Due to the high prevalence of POAG, late detection and serious prognosis for visual functions, this disease occupies a special place in clinical ophthalmology [1].

The proportion of the disease caused by this type of pathology, requiring surgical treatment, is generally small. However, the absolute number of patients with this pathology is quite large: if, in general, in Uzbekistan, the absolute number of patients (2019) with various forms of eye diseases was more than 600 thousand people, then with glaucoma the number of patients was 9,000 [2, 3].

Despite significant advances in the medical treatment of this disease, the percentage of blindness and low vision as a result of glaucoma remains stable and does not tend to decrease. But with the timely detection of the disease, with the help of drug treatment, you can reduce the level of intraocular pressure (IOP).

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The issue of introducing a more effective, but less expensive drug remains relevant [4].

The aim of the study was to conduct a pharmacoeconomic evaluation of Tafluprostvs Travoprost in patients with POAG.

## II. MATERIALS AND METHODS

In this research pharmacoeconomic methods of analysis, in particular relative risk calculation, probability of events calculation, cost-effectiveness analysis was performed in order to achieve the purpose of the investigation.

## III. RESULTS AND DISCUSSIONS

To evaluate the cost-effectiveness of Tafluprost compared with Travoprost in patients with POAG, a Markov analytical model was implemented. The structure of the model was taken from the NICE Guide (2017). Patients were initially classified into glaucoma conditions based on visual field characteristics (Hoddap–Parrish–Anderson criteria) [5].

To monitor patients for more than 3 years, a 1-month cycle was used. During a Markov cycle, members of a cohort may stay at their stage, die, or progress. Because glaucoma can only be prevented from worsening further, none of the cohort members can regress along the clinical path. Based on the HPU (hectopascal unit of pressure) classification system, patients were divided using the mean deviation (MD) value into early (MD less than -6dB), moderate (MD less than -12dB) and severe (MD greater than -12dB). It was believed that the main impact of each strategy was to increase or decrease the risk of developing POAG. However, according to clinical data, the most detailed risk factor for treatment outcomes is adjusting for changes in IOP. To find the relative risk of developing glaucoma of each of the interventions, a systematic search was carried out, moreover, the probabilities of transition between stages were found using another search in the literature. Since the model assumed that an increase in IOP is associated with a further increase in the likelihood of developing glaucoma, this study showed that costs decrease when the progression of the disease is inhibited [6, 7].

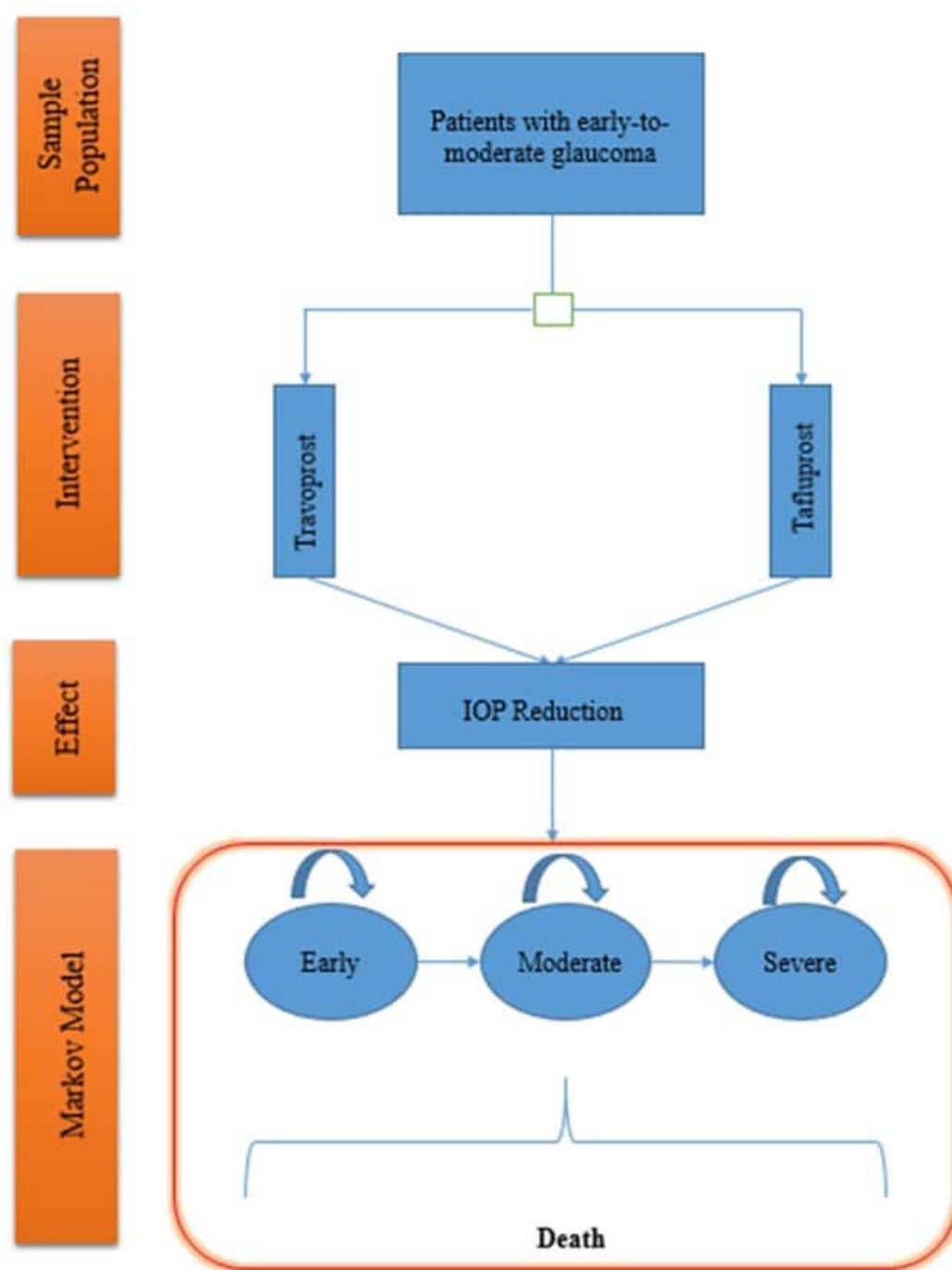


Fig. 1: Initial decision tree integrated with Markov model

Several input parameters were used to populate the model. These baseline data were based on clinical evidence derived from effective evidence review, attempting to broadly allocate resources to guideline updates and supplementary databases. Model inputs have been reviewed and validated by clinical experts. More details on sources - clinical outcomes, utilities and costs are explained below.

Initially, the search was conducted to determine the likelihood of a treatment-related transition during the follow-up period. Since all the data obtained reflected a period of time that exceeded the duration of the cycle in

our model, we converted the information into monthly probabilities (Table 1.). The incremental cost-effectiveness study presents the annual risk of progression to routine glaucoma with moderate to severe clinical treatments. Input parameters were based on 601 patients followed up for 5 years in the Primary Glaucoma Treatment Collaborative Study (PGTCS). Calculation of transition probabilities from early to severe glaucoma was based on various combinations of methods, including natural decline, from the study of early overt glaucoma to the NICE methodology [11]. The study reported transition probabilities by estimating the

number of months it takes a normal patient to change from one state of well-being, then to the next, which was found to decrease in efficiency-adjusted MD effectiveness each month. IOP and age were identified

as significant risk factors. The total mortality of people over 65 years of age was obtained from WHO sources to calculate the Markov model.

**Table 1:** Probabilities of transition between health states of patients with glaucoma

Glaucoma Stages	Average monthly probabilities	Min	Max	Author, Year
P (early to moderate)	0.003779977	0.003023982	0.004535973	Rein et al., 2009 Lichter et al., 2001
P (moderate to severe)	0.003779977	0.003023982	0.004535973	Rein et al., 2009 Lichter et al., 2001
Mortality				
Total mortality of the population over 65 years of age	0.000483333	0.000386667	0.00058	World Health Organization, 2016

Since glaucoma is a chronic disease, it has a great impact on many stages of a patient's life. Although glaucoma eventually leads to permanent blindness, the performance of daily activities and the quality of life recognized by individuals seriously affect health in the early stages of the disease. There are many potential causes of the impact of glaucoma on the patient's quality of life, such as loss of visual field, stress and anxiety due to tests in clinics, impairment and cost of medical care [13]. Many authors have investigated the impact of illness at health stages on the quality of life of patients. Utility values for early, moderate, and severe health conditions in glaucoma were derived primarily from two studies. The first study included a cross-

sectional study of 434 patients with 5 common eye conditions, including glaucoma. Computerized preference scores were used to rate standard utilities from 0 (death) to 1 (excellent health). The second study analyzed the impact of the applied therapy on the utility of patients with glaucoma. The sample population consisted of 225 patients in the same age group as in our evaluation. To the best of our knowledge, a noteworthy finding from this study is that no interference was found between treatment and utility of glaucoma. When reviewing both studies, no rapid discrepancies were found between beneficial outcomes at different stages of glaucoma. Table 2 shows the detailed utility values used in the model.

**Table 2:** Utility values used in the economic model

Health status	Utility	Min.	Max.	Author, Year
Early glaucoma	0.92	0.736	1.104	Lee et al. (2008) and Palette Guedes et al. (2015)
Moderate glaucoma	0.89	0.712	1.068	Lee et al. (2008) and Palette Guedes et al. (2015)
Severe glaucoma	0.86	0.688	1.032	Lee et al. (2008) and Palette Guedes et al. (2015)
Death	0.00			

Cost and resource utilization parameters were obtained from a clinical specialist who works at the Specialized Research Center for Eye Microsurgery in Tashkent.

To calculate the monthly intervention costs, we first derived the annual cost of POAG by multiplying the average unit cost by the expected resource use. Each stage of the disease includes the necessary diagnostic costs, the salaries of medical staff and the weighted cost of medicines. Average prices for prostaglandin analogues and eye drop with beta-blockers were obtained from local pharmacies in Tashkent. After the early stage of glaucoma, monotherapy with Travoprost (Travatan 2.5 ml) or Tafluprost (Teflotan 2.5 ml) was prescribed. However, patients with moderate progression are treated with combined beta-blockers and prostaglandin analogues. The operation is applied on both eyes in a severe condition of glaucoma. In accordance with the recommendations of experts, the frequency of medical diagnoses was set to 4 times a

year, that is, once every three months. In addition, it is estimated that 15 vials of Teflotan 2.5 ml and Travatan 2.5 ml are consumed annually per patient. Meanwhile, the patient is annually prescribed 7 vials of timolol maleate, 5 ml each, and Oftan® Timolol, 5 ml each.

Table 3: The cost of treatment used in the model

The cost of treatment in one case	Medium Cost (UZS)	Minimum Cost (UZS)	Maximum Cost (UZS)
Diagnostics and laboratory tests			
Visometry Test	21,300	14,910	27,690
Simple optical correction	23,000	16,100	29,900
Biomicroscopy	40,000	28,000	52,000
Simple perimetry	23,000	16,100	29,900
The cost of treatment in one case	Medium Cost (UZS)	Minimum Cost (UZS)	Maximum Cost (UZS)
Tonometry	25,000	17,500	32,500
Gonioscopy	30,000	21,000	39,000
Direct ophthalmoscopy	30,000	21,000	39,000
Reverse ophthalmoscopy	40,000	28,000	52,000
Consultation	27,000	18,900	35,100
<b>Total diagnostic and lab costs per visit</b>	<b>1,037,200</b>	<b>726,040</b>	<b>1,348,360</b>
Care cost per visit (20 min)	2,536	1,775	3,296
Doctor cost per visit (20 min)	4,620	3,234	6,006
Total payroll costs fee	<b>7.156</b>	<b>5.009</b>	<b>9.302</b>

Table 4: Cost of eye drops used in the model

The cost of treatment in one case	Medium Cost (UZS)	Minimum Cost (UZS)	Maximum Cost (UZS)
Eye drops			
Taflatan 2.5ml	90,000	63,000	117,000
Travatan 2.5ml	140,000	98,000	182,000
Timolol maleate 5ml	30,000	21,000	39,000
Oftan® Timolol 5ml	25,000	17,500	32,500
Annual cost of intervention per patient	early glaucoma (UZS)	Moderate glaucoma (UZS)	Severe glaucoma (UZS)
Tafluprost	2,415,824	2,800,824	5,815,824
Monthly intervention cost per patient	<b>201.319</b>	<b>233.402</b>	<b>484.652</b>
Travoprost	3,165,824	3,550,824	6,565,824
Comparator monthly cost per patient	<b>263.819</b>	<b>295.902</b>	<b>547.152</b>

Cost-effectiveness study used actual numbers or averages as model parameters. This strategy gives the best estimate of the cost-effectiveness of the Tafluprost intervention, but does not take into account the uncertainty about model inputs or the likelihood of a different sequence of events. A widely used cost-effectiveness measure is to apply the incremental cost-effectiveness ratio (ICER) when comparing Tafluprost and Travoprost eye drops.

During the cost-effectiveness analysis, which gives the best estimate of the cost-effectiveness of Tafluprost, a sensitivity analysis was performed to assess the vulnerability of the model and clinical incidents. To assess the effect of changing one parameter or the parameter that had the greatest impact on the model results, we performed a one-sided sensitivity analysis. When performing a one-sided sensitivity analysis, the valid ranges of the model input data were used. This made it possible to evaluate the individual impact of model inputs on the results. To conduct the Monte Carlo simulation, the model was run on a cohort of 1000 patients and the selected inputs

were randomly selected based on the assigned distribution. The results were presented in Table 5, then looking at the cost-effectiveness threshold at the country level, the prospects for the appropriateness of the proposed intervention were assessed.

Table 5: Input range for one-sided sensitivity analysis

Variable	Range	
	High	Low
Overall mortality in patients over 65 years of age	0.00058	0.00039
Cost discount rate	0.003	0.002
Results discount rate	0.003	0.002
The cost of a severe stage per month Tafluprost	\$630,047.60	\$339,256.40
The cost of a severe stage per month Travoprost	\$711,297.60	\$383,006.40
Likelihood of switching from moderate to severe glaucoma with Tafluprost	0.004535973	0.003023982
Probability of going from early to moderate glaucoma with Tafluprost	0.004535973	0.003023982
Relative risk of Travoprost	1.235337423	0.823558282
Health Benefits of Early Glaucoma	1.104	0.736
Moderate stage cost per month Tafluprost	\$303,422.60	\$163,381.40
Health Benefits in Severe Glaucoma	1.032	0.688
Early-stage cost per month Tafluprost	\$261,714.27	\$140,923.07
Cost of the moderate stage per month Travoprost	\$384,672.60	\$207,131.40
Early-stage cost per month Travoprost	\$342,964.27	\$184,673.07
Benefits of Moderate Health in Glaucoma	1.068	0.712

The results of a one-way sensitivity analysis suggested that the variable that strongly influenced the economic model was the utility of the moderate stage of glaucoma. In absolute terms, when the QALY health utility of moderate glaucoma declined, ICER increased

nearly 6-fold. In addition, when the risk of spending on Travoprost in early to moderate glaucoma was modified, their ICER showed balanced volatility for both parties per QALY. The results of other models are relatively less sensitive than the above parameters (Fig. 1).

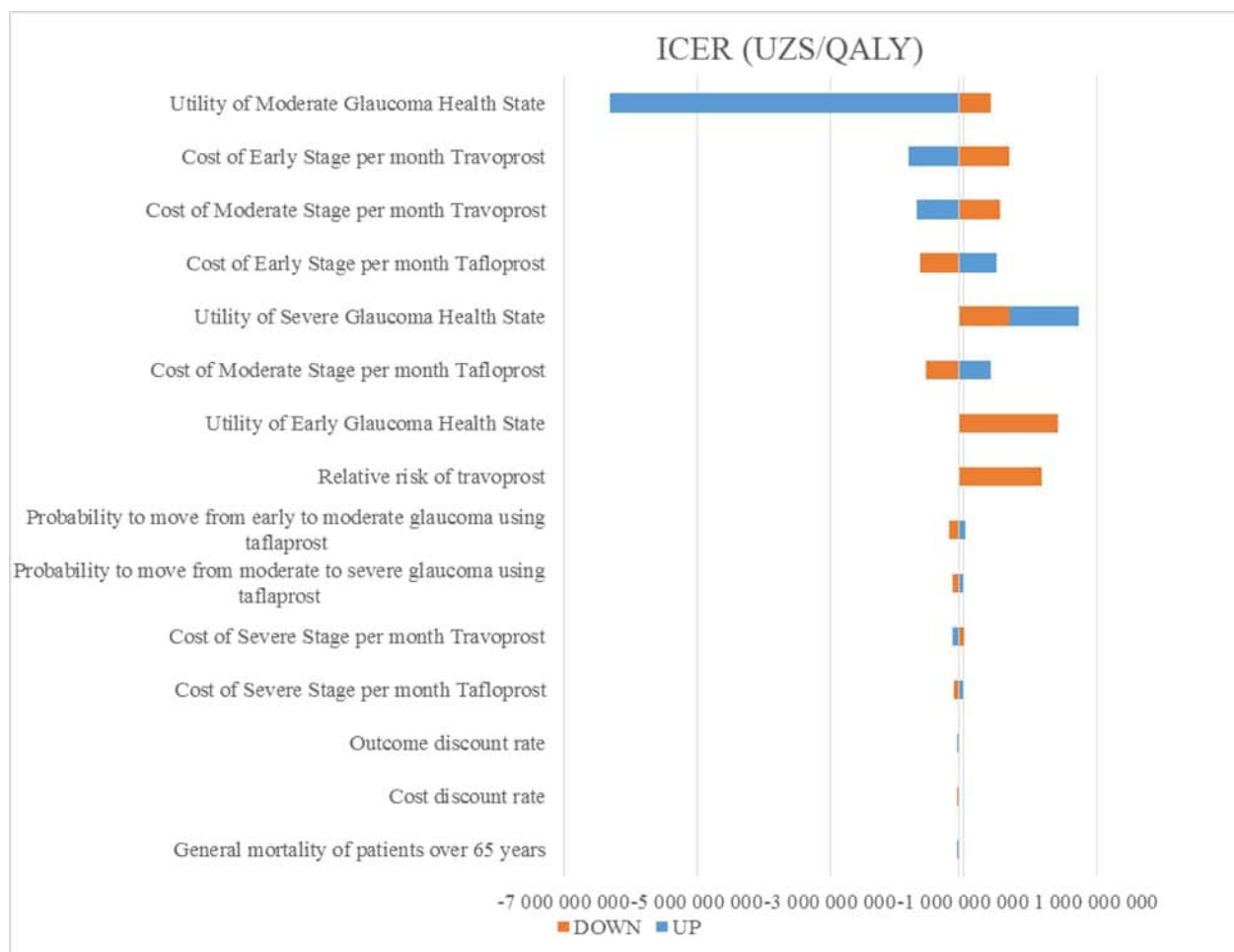


Fig. 1: Results of One-Way Sensitivity Analysis



The results from the cost-effectiveness analysis for the reference case are presented in Table 6. On average, the new intervention dominated by Tafluprost is less costly and clinically more effective than the

comparator drug (Travoprost). We calculated that a savings phenomenon could be observed in the treatment of patients with POAG (compared to the Brown study).etal.), so ICER was negative.

**Table 6:** Results of cost-benefit analysis

Strategy	Average total cost	Average overall effect, QALYs	ICER UZS/QALY
Travoprost	9,721,341	30.878	-1 069 362
Tafluprost	7,582,616	30.880	

$$ICER = (7\,582\,616 - 9\,721\,341) / (30.880 - 30.878) = -1\,069\,362 \text{ UZS/QALY}$$

that in the treatment of glaucoma, when using Tafluprost, you can save 2.138.725 UZS (Table 7).

Next, we performed a budget impact analysis. According to the results of the analysis, it can be seen

**Table 7:** The results of the “influence on the budget”

Transition in treatment glaucoma on Tafluprost with Travoprost	Payment	the effect influence on budget, sum	A comment
	9,721,341 - 7,582,616	= 2,138,725	Saving funds

The cost-effectiveness analysis based on the model showed that the treatment of patients with POAG with Tafluprost has a dominant advantage over the reference drug Travoprost. Despite marginal improvement in quality of life, the Tafluprost intervention resulted in cost savings due to less resource use. However, it is interesting to note that our results are not consistent with a recent US study comparing several prostaglandin analogs in the treatment of patients with glaucoma. Brown Research et al. (2019) showed that Tafluprost is more costly and effective than Travoprost. The additional allowance for the target group was about US\$214,828. However, since the economic study took into account cost parameters related to a developed country such as the United States, it is inappropriate to compare with our findings from the perspective of Uzbekistan. Our analysis shows that the exclusive use of Tafluprost rather than Travoprost in the treatment of patients with glaucoma prevents additional economic burden. Under these model assumptions, it has been calculated that delaying progression in early states of glaucoma may prevent patients from taking additional glaucoma medications and even eye surgery in advanced stages of the disease. Based on this, we can assume that Tafluprost would be the most practical option in the reference center environment for the treatment of glaucoma, which would save money for the healthcare system in Uzbekistan.

The study has many strengths. A short analytical decision tree and a Markov model were used to collect epidemiological, clinical, resource utilization, and outcome estimates. The model included the likelihood of glaucoma progressing to advanced stages. In addition, these stages reflect both the clinical and

economic consequences of glaucoma. The literature used to derive the specific parameters in our model is based on the sufficient size of the observation period and the target population. We used specific cost data for Uzbekistan, which was unprecedented in this area for individual interventions in the treatment of patients with POAG.

## IV. CONCLUSIONS

According to the calculated results of pharmacoeconomic analyzes, Tafluprost was relatively less expensive and more clinically effective than Travoprost in patients with primary open-angle glaucoma. Alternative treatment with Tafluprost contributes to savings in the overall treatment procedure. Therefore, we recommend adding Tafluprost to the list of essential medicines.

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## Ophtalmologic Cystinosis

By Fiqhi Aissam

*Introduction-* Cystinosis is a very rare lysosomal, autosomal recessive disease (1/200,000 births) caused by a mutation in the CTNS gene (Chz 17) encoding a protein called cystinosine. The intralysosomal accumulation of cystine induces the formation of insoluble crystals responsible for progressive multiple organ failure. Cystinous nephropathy is manifested by failure to thrive, Fanconi syndrome, damage to the renal glomerulus and manifestations affecting other organs appearing as early as 6 to 12 months of life. The specific treatment for cystinosis is cysteamine. The management is multidisciplinary.

We report the case of a boy, aged 6 years, followed for cystinosis diagnosed at the age of 18 months with polyuropolydipsic syndrome. The patient has been treated with oral cysteamine (Cystagon) since the age of 2 years.

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Cystinosis is a very rare lysosomal, autosomal recessive disease (1/200,000 births) caused by a mutation in the CTNS gene (Chz 17) encoding a protein called cystinosine. The intralysosomal accumulation of cystine induces the formation of insoluble crystals responsible for progressive multiple organ failure. Cystinosis nephropathy is manifested by failure to thrive, Fanconi syndrome, damage to the renal glomerulus and manifestations affecting other organs appearing as early as 6 to 12 months of life. The specific treatment for cystinosis is cysteamine. The management is multidisciplinary.

We report the case of a boy, aged 6 years, followed for cystinosis diagnosed at the age of 18 months with polyuropolydipsic syndrome. The patient has been treated with oral cysteamine (Cystagon) since the age of 2 years.

On the ophthalmological level, his corrected visual acuity is evaluated at 10/10 P2 ODG, and the intraocular pressure is at 12 mmHg in both eyes. The main complaint of this patient is photophobia. He is treated with cysteamine eye drops (Cystadrops®) at a dosage of 4 drops per day and additional treatment with artificial tears. With the slit lamp, we find a corneal cystinosis of grade 1.50 according to the classification of Gahl (figure 1): deposits of birefringent spindle-shaped crystals accumulating in the corneal stroma, and progressing from the anterior stroma to the endothelium. The appearance is sparkling and multicolored on biomicroscopic examination.

The severity of the corneal involvement can be assessed by corneal OCT.

The fundus does not show retinopathy. Cystinosis retinopathy is very inconstant. Crystal deposits are sometimes found all over the retina, associated with depigmentation and which can progress to retinal atrophy. It can be explored by retinal angiography. The crystals can also deposit on the conjunctiva, iris, ciliary body, anterior lens capsule, choroid or optic nerve and cause various rarer manifestations, such as glaucoma by closing the angle, papillary edema or visual field changes.

Ophthalmologic monitoring for cystinosis should be annual, with visual acuity assessment, Gahl score estimate supplemented by OCT imaging of the cornea, measurement of intraocular pressure and fundus examination.

This case illustrates a well-followed patient who is coping well with his illness.

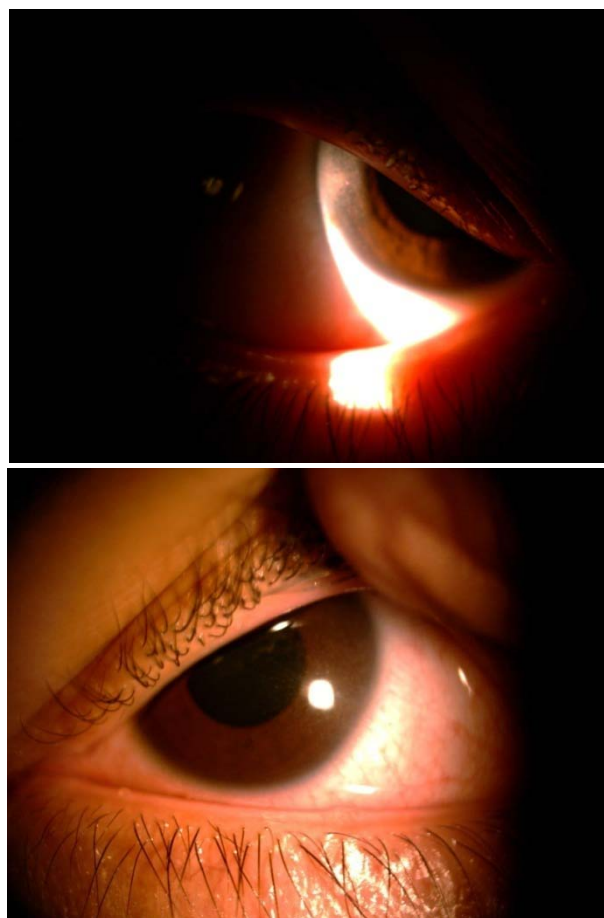


Figure 1: Corneal crystal deposits.

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# Using the Method of Mathematical Planning of the Experiment in the Development of an Optimal Technology for Obtaining Dry Extract from the Chological Collection "Triflos"

By Nargiza Abdumajidovna Abdurakhmanova & Yokut Saidkarimovna Karieva

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**Abstract-** Research is carried out on the development of technology for the dry extract of the choleretic collection "Triflos" by the method of mathematical planning of the experiment. In this case, the method of a four-factor experimental plan based on a 5x5 Greek-Latin square is used. The use of short-term ultrasonic exposure to intensify the extraction of target groups of biologically active substances is scientifically substantiated. The proposed technology is tested in industrial conditions. The dry extract yield was 21%.

**Keywords:** *choleretic collection "Triflos", dry extract, the degree of grinding of raw materials, extractant, temperature, hydromodule, circulating extraction, ultrasound.*

**GJMR-B Classification:** DDC Code: 641.4 LCC Code: TX603



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# Using the Method of Mathematical Planning of the Experiment in the Development of an Optimal Technology for Obtaining Dry Extract from the Chological Collection "Triflos"

Nargiza Abdumajidovna Abdurakhmanova<sup>a</sup> & Yokut Saidkarimovna Karieva<sup>o</sup>

**Abstract-** Research is carried out on the development of technology for the dry extract of the choleretic collection "Triflos" by the method of mathematical planning of the experiment. In this case, the method of a four-factor experimental plan based on a 5x5 Greek-Latin square is used. The use of short-term ultrasonic exposure to intensify the extraction of target groups of biologically active substances is scientifically substantiated. The proposed technology is tested in industrial conditions. The dry extract yield was 21%.

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## 1. INTRODUCTION

The Decree of the President of the Republic of Uzbekistan Shavkat Mirziyoyev "On measures to further improve the provision of the population with medicines and medical products" dated October 31, 2016 is an important factor in expanding the scope of work in the pharmaceutical industry. This document outlines important tasks for increasing the localization of pharmaceutical production by expanding the use of local raw materials, in particular, medicinal plants, and improving the delivery of quality medicines to the population at affordable prices.

One of the tasks provided for in the Decree of the President of the Republic of Uzbekistan dated April 10, 2020 No. 4668 "On additional measures for the development of traditional medicine in the Republic of Uzbekistan" and No. PP-4670 "On measures for the protection, cultural cultivation, processing of wild medicinal plants and rational use of available resources" is the organization of cultivation and harvesting of plants and raw materials of non-plant species used in traditional medicine, conducting laboratory and scientific research in this direction, increasing the export potential of the industry, as well as integrating education, science and production processes. In this regard, the Tashkent Pharmaceutical Institute is carrying out consistent work to ensure the implementation of the priority tasks outlined in the resolutions.

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One of the promising directions in the field of drug development is the creation of phytopreparations for the treatment and prevention of these diseases. More active usage of phytopreparations, both alone and in combination with synthetic drugs, depending on the severity and nature of diseases, will contribute to the use of drug-saving technologies in clinical practice. Natural biologically active substances of plants are evolutionarily closer to the human body than synthetic ones, they are easily included in metabolic processes and have practically no side effects, and many of them are precursors of physiologically active substances (hormones, mediators). Recent studies have shown that the healing properties of medicinal plants depend on the harmonious interaction of all active substances, which together have a broader effect than individually.

In this regard, studies on the conversion of collections and individual plants used in the form of infusions and decoctions into total preparations-extracts are very promising, since the possibility of their wider use is limited by the imperfection of the dosage form [1,2].

Summarizing the above, we can conclude that the creation of medicines and dietary supplements based on medicinal plant materials using a resource-saving extraction method (due to the maximum depletion of raw materials) is timely and relevant and corresponds to the social order of clinical medicine.

Given the above, the Tashkent Pharmaceutical Institute has developed a choleretic collection "Triflos", consisting of the following types of herbal medicinal raw materials: flowers of tansy false yarrow, flowers of chamomile, yarrow herb. To date, studies are carried out on its standardization, the establishment of quality standards, as well as the study of specific activity and safety. Cholagogue collection when tested on laboratory rats showed more than 50% increase in bile secretion [3]. The pharmacotherapeutic effects of this collection, which mainly consist in the regulation of phospholipid metabolism in the liver and the normalization of bile flow due to the antispasmodic effect on the sphincter of the gallbladder, according to the opinion prevailing in the literature, is determined by the presence of a number of

phenolcarboxylic acids and flavonoid compounds. So, in the composition of the collection components, along with essential oils, terpenoids, bitter and tannins, resins, organic acids and mineral salts, the presence of chlorogenic, rosmarinic, caffeic, ferulic acids and their corresponding glycosidic derivatives, as well as flavonoids: quercetin, luteolin, acacetin, apigenin and their glycosides. These literature data were confirmed by us experimentally [4].

The purpose of our research is to develop an optimal technology for obtaining a dry extract from this collection using the method of mathematical planning of the experiment.

## II. EXPERIMENTS AND ITS RESULTS

Currently, there are a large number of various extraction schemes designed to increase the yield of active substances, ensure maximum depletion of raw materials and enrichment of the extract with target

biologically active substances (BAS). These extraction schemes also include factors that affect the completeness of extraction of biologically active substances, such as the nature and concentration of the extractant, the ratio of raw materials and extractant, temperature, type of extraction, etc. [5-18].

In order to facilitate the laborious process of finding the optimal conditions for extraction, we decided to apply the method of mathematical planning. Based on the characteristics of the technology for obtaining a dry extract, we chose a four-factor experimental plan based on a 5x5 Greek-Latin square. At the same time, the influence of the degree of grinding of raw materials (factor A), the concentration of ethyl alcohol (factor B), the hydromodulus (factor C) and the temperature regime (factor D) on the completeness of the yield of the sum of flavonoids in terms of quercetin in extraction is studied [19].

Factors and their levels are given in Table 1.

Table 1: Factors and their levels used in the experiment

Factor and its levels	Factor value	Factor and its levels	Factor value
<i>The degree of grinding of raw materials (factor A)</i>		<i>Ethyl alcohol concentration (factor B)</i>	
a <sub>1</sub>	2-4 mm	b <sub>1</sub>	50%
a <sub>2</sub>	5-7 mm	b <sub>2</sub>	60%
a <sub>3</sub>	8-10 mm	b <sub>3</sub>	70%
a <sub>4</sub>	11-13 mm	b <sub>4</sub>	80%
a <sub>5</sub>	14-16 mm	b <sub>5</sub>	90%
<i>Hydromodulfactor (factor C)</i>		<i>Temperature factor (D)</i>	
c <sub>1</sub>	1:5	d <sub>1</sub>	40°C
c <sub>2</sub>	1:10	d <sub>2</sub>	50°C
c <sub>3</sub>	1:15	d <sub>3</sub>	60°C
c <sub>4</sub>	1:20	d <sub>4</sub>	70°C
c <sub>5</sub>	1:25	d <sub>5</sub>	80°C

The 5x5-experiment plan and the results of determining the quantitative content of biologically active substances (the sum of flavonoids in terms of quercetin) are presented in Table 2.

Table 2: The yield of the sum of flavonoids in terms of quercetin in the extract in a four-factor plan 5x5 with three repeated experiments, %

Factor A	Factor B					Totala <sub>i</sub>
	b <sub>1</sub>	b <sub>2</sub>	b <sub>3</sub>	b <sub>4</sub>	b <sub>5</sub>	
a <sub>1</sub>	C <sub>1</sub> d <sub>1</sub>	c <sub>2</sub> d <sub>2</sub>	c <sub>3</sub> d <sub>3</sub>	c <sub>4</sub> d <sub>4</sub>	c <sub>5</sub> d <sub>5</sub>	23,78
	1,22	2,15	2,14	1,36	1,04	
	1,28	2,09	2,17	1,41	1,09	
	1,21	2,19	2,11	1,33	0,99	
a <sub>2</sub>	3,71	6,43	6,42	4,1	3,12	27,7
	c <sub>2</sub> d <sub>3</sub>	c <sub>3</sub> d <sub>4</sub>	c <sub>4</sub> d <sub>5</sub>	c <sub>5</sub> d <sub>1</sub>	c <sub>1</sub> d <sub>2</sub>	
	2,15	1,89	2,02	1,56	1,62	
	2,11	1,93	2,06	1,52	1,59	
a <sub>3</sub>	2,17	1,84	1,97	1,6	1,67	23,8
	6,43	5,66	6,05	4,68	4,88	
	c <sub>3</sub> d <sub>5</sub>	c <sub>4</sub> d <sub>1</sub>	c <sub>5</sub> d <sub>2</sub>	c <sub>1</sub> d <sub>3</sub>	c <sub>2</sub> d <sub>4</sub>	
	1,3	1,55	1,76	1,67	1,62	
a <sub>4</sub>	1,34	1,61	1,71	1,74	1,67	23,8
	1,26	1,52	1,8	1,66	1,59	
	3,9	4,68	5,27	5,07	4,88	
	c <sub>4</sub> d <sub>2</sub>	c <sub>5</sub> d <sub>3</sub>	c <sub>1</sub> d <sub>4</sub>	c <sub>2</sub> d <sub>5</sub>	c <sub>3</sub> d <sub>1</sub>	
a <sub>4</sub>	1,3	1,69	1,56	1,5	1,24	23,8

a <sub>5</sub>		1,34	1,65	1,61	1,52	1,2	21,85
		1,26	1,73	1,51	1,47	1,27	
		3,9	5,07	4,68	4,49	3,71	
		c <sub>5</sub> d <sub>4</sub>	c <sub>7</sub> d <sub>5</sub>	c <sub>2</sub> d <sub>7</sub>	c <sub>3</sub> d <sub>2</sub>	c <sub>4</sub> d <sub>3</sub>	
a <sub>5</sub>		1,01	1,24	1,76	1,37	1,57	20,87
		1,09	1,29	1,73	1,41	1,59	
		1,02	1,18	1,77	1,32	1,52	
		3,12	3,71	5,26	4,1	4,68	20,87
Total	B <sub>i</sub>	21,06	25,55	27,68	22,44	21,27	118
	C <sub>k</sub>	22,05	27,49	23,79	23,41	21,26	
	D <sub>l</sub>	22,04	24,58	27,67	22,44	21,27	

Before carrying out the analysis of variance, the homogeneity of the variance was checked using the Cochran test. The tabular value of the Cochran test for f1=2 and f=25 is 0.22, i.e., uexp, equal to 0.0709, is

less than the tabular one, which confirms the equal accuracy of the experiments. Analysis of variance of the obtained results is presented in Table 3.

**Table 3:** Dispersion analysis of experimental data to determine the yield of the total flavonoids in the obtained extracts

Source of dispersion	Number of degrees of freedom (f)	Sum of squares (SS)	Mean squares (MS)	F <sub>expert</sub>	F <sub>0,05</sub>	Hypothesis
Factor A	4	2,245	0,56125	328,0885	2,56	a≠0
Factor B	4	1,82652	0,45663	266,931	2,56	b≠0
Factor C	4	1,538827	0,384707	224,887	2,56	c≠0
Factor D	4	1,782227	0,445557	260,4579	2,56	d≠0
Remainder	8	0,15216	0,01902	11,11847	2,13	res≠0
Errorinsidecell	50	0,085533	0,001711			
Totalamount	74	7,630267				

The obtained values Fexp>Ftabl, which indicates the statistical significance of all four studied factors. The value of Fres.in.cell indicates the presence of an interaction between the factors.

Using Duncan's multiple rank test, the differences in the average values of the data on the yield of the total flavonoids in the obtained extracts are studied. It is established that according to the influence of the degree of dispersion of plant raw materials on the response, they can be arranged in the following row: a1 = a2 = a3 > a4 > a5, i.e. the optimal degree of grinding of raw materials, providing the maximum yield of the sum of flavonoids, are 2-4 mm, 5-7 mm and 8-10 mm. However, taking into account the fact that the excessive dispersity of plant raw materials will ensure the release of not only biologically active, but also ballast substances, which, accordingly, will lead to a contaminated extract, we decided to use raw materials in further studies, the dimensions of which are in the range of 5-7 mm [20, 21].

The influence of the next studied factor - the concentration of ethanol used as an extractant, can be arranged in the following row: b2 = b3 > b4 > b5 > b1, Thus, ethanol at a concentration of 60% and 70% is the optimal extractant. For the purpose of economic feasibility, our choice was stopped at 60% ethyl alcohol.

It is known that the ratio of vegetable raw materials and extractant has a significant impact on the yield of biologically active substances in the obtained extracts. This was confirmed by the results of our mathematical planning. It was found that almost the same output with a slight difference was obtained with a hydromodulus of 1:10, 1:15, 1:20. In order to save the extractant, we recommend the use of a hydromodule equal to 1:10.

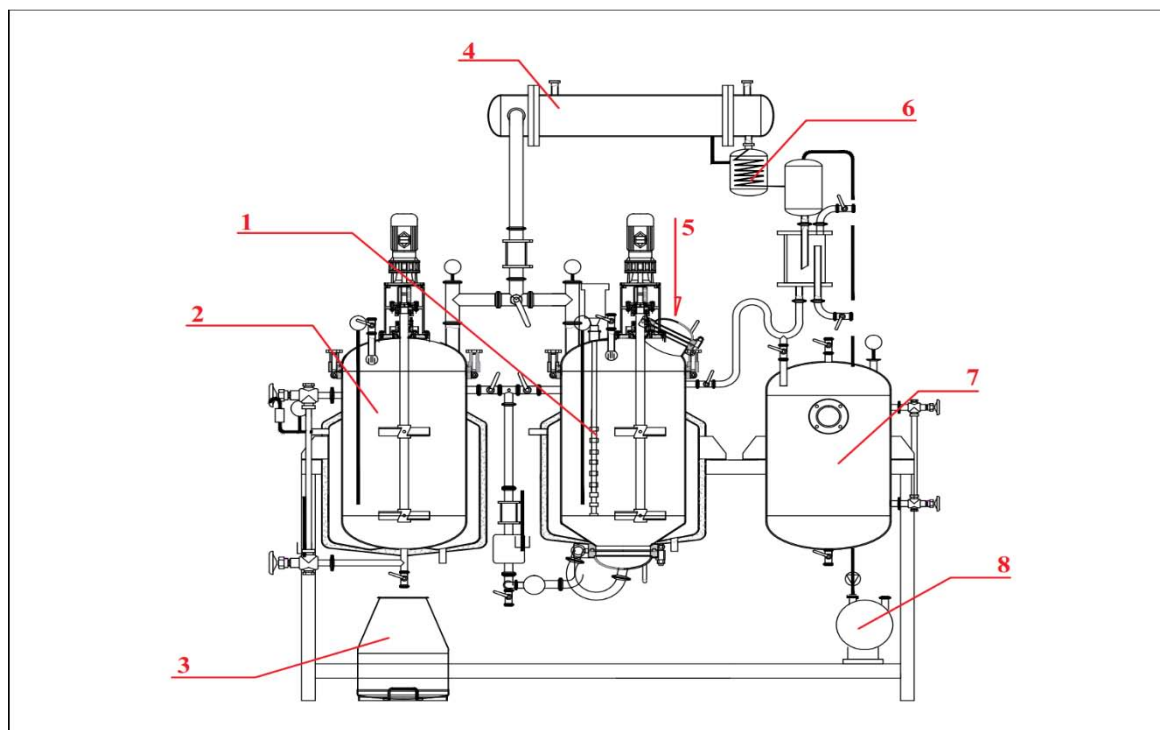
The application of the Duncan rank criterion also helped to reveal the influence of the temperature factor on the yield of the sum of flavonoids, this series can be represented as follows: d2 = d3 > d4 > d1 > d5, thus, the temperature of 500C was chosen as the optimal one.

An analysis of domestic and foreign literary sources indicates that the short-term use of ultrasound in the extraction of plant materials stimulates the release of biologically active substances [22, 23]. Given the above, we carried out extraction from the composition of medicinal raw materials, culminating in 10 minutes of ultrasonic exposure. At the same time, an increase in the yield of the total flavonoids in terms of quercetin is observed from 2.34% to 2.6% (1.11 times).

Thus, the proposed technology for obtaining a dry extract from the Triflos choleric collection is tested under industrial conditions at BALZAM LLC.

The industrial extractor, on which the proposed technology was tested, consists of:

- 1) Main tank. This is the extraction tank, which is the main element of this system. It mainly serves to extract biologically active substances from medicinal plant materials;
- 2) A condenser, which serves to cool and return the condensed liquid to the extraction tank;
- 3) A vacuum condenser, which serves to concentrate and collect the resulting extract;
- 4) A tank for collecting the extractant (used as a collection) (Fig. 1).



**Fig.1:** Scheme of the extractor "RUIAN XUANLI MASHINERY TANK". 1-extractor with built-in ultrasonic device; 2-vacuum concentrator; 3-collection; 4- capacitor; 5- loading hopper; 6- refrigerator; 7- tank for alcohol recovery; 8- vacuum pump

This equipment can carry out several operations simultaneously; control and regulation of the temperature regime of the extraction process, use ultrasound, create a vacuum, condense the extractant, recover alcohol, etc.

To obtain a dry extract, medicinal plant raw materials, crushed to a size of 5-7 mm, are weighed and mixed in the ratio: tansy flowers - 15 parts, chamomile flowers - 10 parts, yarrow herb - 10 parts. The mixture of vegetable raw materials was loaded into a special container and soaked with half the amount of 60% ethyl alcohol until a "mirror surface" is formed, left for 24 hours. After soaking, the soaked raw material is transferred to the extractor and 60% ethanol was added, bringing the raw material-extractant ratio to 1:10, the mixture was heated to 50°C, and circulation extraction was carried out in the RuianXuanli Machinery Tank extractor. Next, ultrasonic extraction is performed for 10 minutes. After the vacuum extraction process is completed, the liquid extract is pumped through the filter into the reactor and left for 24 hours to settle. Purification of the distillation residue of the water-alcohol extract is carried out by filtration.

Then, the extractant was distilled off in the reactor using vacuum for 3 hours. The extract remaining after distillation of the extractant was spray dried in a high-speed spray dryer "LPG-15 Spray Dryer" (manufactured by RuianXuanli Machinery Co., LTD). (Fig. 2).



Fig. 2: Spray dryer "LPG-15 HIGH SPEED SPRAY DRYER"

As a result of the research, a light brown dry extract with a weak specific odor and a bitter taste is obtained. The dry extract yield is  $21 \pm 1.53\%$ .

### III. CONCLUSIONS

1. Using the method of a four-factor experimental plan based on a 5x5 Greek-Latin square, the choice of the degree of grinding of raw materials, the concentration of ethanol, the ratio of raw materials and extractant, the temperature regime in the development of the technology of dry extract of the choleretic collection "Triflos" is carried out. The results of dispersion analysis shows that the most complete yield of the sum of flavonoids is observed with the following indicators of the above factors: dispersion of raw materials -5-7 mm, 60% ethyl alcohol, hydromodulus 1:10, temperature 500C.
2. The expediency of using ultrasonic treatment for a more complete recovery of target biologically active substances is scientifically substantiated.
3. This technology of circulating extraction using ultrasound is tested in industrial conditions. A dry extract is obtained, the yield of which was 21%.

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# Water Contamination by Nitrates and its Thyroid Disruptive Action. Bioassay on *Xenopus Laevis*

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**Objectives:** To assess the thyroid disrupting action of groundwater contaminated with nitrates and arsenic, by means of a bioassay of chronic toxicity in *Xenopus laevis* larvae.

**Methods:** Three experimental groups immersed in water: Control group (C) (n=13) filtered drinking water, Exposed group (E) groundwater (n=18) and Positive Control group (PC) (n=18) filtered drinking water added with 0,007 mg/l of potassium perchlorate. A water physicochemical analysis was performed. The duration of metamorphosis stages, total body length, mortality per group, weight and height were morphologically evaluated. The colloid volume, degrees of hyperplasia, and height of the follicular epithelium of the thyroid gland were histologically evaluated. At molecular level, NIS thyroid symporter protein expression was measured.

**Keywords:** endocrine disruptors - xenopus - groundwater - nitrates – thyroid.

**GJMR-B Classification:** DDC Code: 174.95 LCC Code: Q175.35



*Strictly as per the compliance and regulations of:*



# Water Contamination by Nitrates and its Thyroid Disruptive Action. Bioassay on *Xenopus Laevis*

María Fernanda Modarelli <sup>α</sup>, Rodrigo Miguel Bilbao <sup>σ</sup> & Osvaldo Juan Ponzo <sup>ρ</sup>

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**Results:** The groundwater physicochemical analysis showed the presence of nitrates (values between 24 and 83 mg/l) and arsenic (0.05 mg/l). Prometamorphosis was longer in group E Vs C (p<0.0001). In PC only three animals completed this stage (p<0.0001). Weight was in increasing order E<PC<C and height E<PC<C (p<0.05). Mortality recorded per group was: 10% in group E exclusively (p<0.0001). Changes could be noticed in the thyroid glandular histoarchitecture at stage 58NF: hyperplasia grade 1 in C, grade 2 in E and PC (p<0.0001). The colloid area and the height of the follicular epithelium were in increasing order PC<C<E (p<0.0001). The level of expression in the larval thyroid tissue of NIS symporter was in increasing order C<PC<E (p<0.0001).

**Discussion:** Changes observed in the thyroid gland, as well as the morphological alterations, of *Xenopus laevis* larval development at stage 58NF, could be related to the presence of nitrates and arsenic in the groundwater which cause a synergic disruptive action on the thyroid.

**Keywords:** endocrine disruptors - *xenopus* - groundwater - nitrates – thyroid.

## I. INTRODUCTION

Endocrine disruptors (EDs) affect the normal function of the endocrine system, by interfering with the synthesis, storage, transport, circulating levels, peripheral action and catabolism of hormones. Thyroid disruptors (TDs) are a group of chemical substances that affect the hypothalamus-pituitary-thyroid (HPT) axis in different ways, for example through their capacity to decrease the circulating levels of thyroid hormones (Brucker-Davis F. 1998), or acting directly on their receptors as well as on the enzyme or plasmatic carriers which play a significant role in the mediation of its action (Howdeshell KL. 2022) in humans as well as in animals (Colborn T. et al. 1993).

Contaminated groundwater could vehicle different EDs as nitrates, perchlorates and thiocyanates, among others (Zewdie T. et al 2010). Endemic areas of hypothyroidism and goiter with no iodine deficit have been described, being a probable cause the presence of EDs in the water drank by the population living in those areas (Andrada I. et al. 2009).

The correct thyroid function involves a proper activity of the sodium/iodide symporter (NIS) at thyroid follicular cells. In mammals and amphibians, thyrotropin (TSH) stimulates NIS expression being involved in this transcription factors as PAX-8, TFF-1 and TTF-2 (Dohan O. et al. 2003). The NIS symporter inhibition interferes with iodine uptake, decreasing the synthesis of triiodothyronine (T<sub>3</sub>) and thyroxine (T<sub>4</sub>) which results in a TSH increase. Consequently, a higher stimulation in an attempt to compensate the hormone synthesis, leads to the development of goiter (Crofton K. et al. 2005).

During the spontaneous amphibian metamorphosis, the NIS mRNA expression is low in pre-metamorphous tadpoles and it increases throughout prometamorphosis, as the same time as the increase of mRNA expression of TSH beta subunit (TSHb) at tadpole hypophysis, thus suggesting a TSH regulation in the NIS expression (Opitz R. et al. 2006). Moreover, thyroid hormones play a fundamental role in amphibian and on human fetus development (Zoeller RT. et al. 2004).

Amphibian larvae are used as bioassays for being highly sensitive to the action of different substances present in water, even in LOAEL (Lowest Observed Adverse Effect Level) and NOAEL (No Observed Adverse Effect) concentrations (FETAX.

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2000). The metamorphosis of these animals is a process that depends on thyroid hormones, being this influence higher during the prometamorphosis and climax stages (Tietge JE. et al. 2005). In a short period of time larvae suffer structural, physiological, biochemical and behavioral transformations due to  $T_4$  action and to the conversion of  $T_4$  into  $T_3$  in the target organs. These alterations are accompanied by changes in thyroid gland volume, height of follicular epithelium, colloid reabsorption level and iodine uptake. These processes are regulated by  $T_4$  which secretion increases at prometamorphosis onset and continues rising up to the end of metamorphosis (Miranda LA. 1995), to achieve tail resorption. In the case of *Xenopus laevis* the thyroid gland becomes operational at prometamorphosis onset (Nieuwkoop PD et al. 1967), similar to what happens with other anurans (Saxen L. et al. 1957).

Disorders which involve iodine transport and lead to a change in the thyroid hormone synthesis, may cause changes in the growth and metabolism during amphibian metamorphosis, as well as in humans (Shi YB. et al. 1996). Because of this, *Xenopus laevis* larvae can be used as a biologic model to study "in vivo" the biological effect of endocrine disruptors (EDs), by evaluating the morphological and functional changes normally induced by thyroid hormones (THs).

This study proposes that the immersion of *Xenopus laevis* larvae "in vivo" in groundwater could cause morphological, histological and biomolecular changes which are the result of the presence of endocrine disruptors (EDs) in such water under study (Modarelli MF. and Ponzo OJ. 2018).

## II. METHODS

We experimented with *Xenopus laevis* larvae from the Endocrinology Laboratory of the Institute of Physiology, School of Medicine, University of Buenos Aires. Larvae used for this experiment were obtained after amplexus (of adult specimens), of only one spawn. Samples from healthy specimens with no malformations and with a homogenous size were selected in accordance with ANFICOR guidelines (Herkovits J. et al. 1999). Selected specimens were placed in transparent containers; one larva by each 500 cm<sup>3</sup> of water, held in stable conditions on a 12 h light: 12 h darkness photoperiod, temperature: 22° C ± 2° C and pH: 7.2 to 8, in filtered drinking water with extraction of chlorine by carbon filter, and were fed ad libitum with balanced feeds (Sera Micron). To reduce specimen stress, the same person changed the water and controlled larvae each 48 h. The protocol was approved by institutional animal care and use committee (CICUAL/UBA: 0003598/2013. Res. 700).

### a) Experimental Design

Larvae were divided into 3 treatment groups: a) Control group (C) (n=13) immersed in filtered drinking water; b) Exposed group (E): immersed in 30-meter-depth well groundwater from the southern suburbs of Buenos Aires, Argentina (n=18); Positive Control group (PC): immersed in filtered drinking water added with 0,007 mg/l potassium perchlorate (KClO<sub>4</sub>) as NIS-inhibiting thyroid disruptor (n=18). All animals underwent 70-day-period treatment. Partial study cuts were made at premetamorphosis, prometamorphosis and climax stages, using Nieuwkoop and Faber (NF) criteria (Nieuwkoop PD et al. 1967) to determine those different stages. Mortality per group was recorded and morphological changes in larvae such as: total time of metamorphosis, time of premetamorphosis, prometamorphosis, climax, weight and height (Organization for economic Co-Operation and Development. (OECD). 2004) were analyzed. Moreover, thyroid gland histological changes were evaluated like colloid area, height of follicular cell, number of follicles per field, hyperplasia and hypertrophy (Grim C. 2007). Finally, NIS symporter protein expression in the thyroid tissue was studied using Western Blot technique.

### b) Histological Technique

Larvae were sacrificed by immersion in MS222 (200mg/l) solution for later histological and biomolecular evaluation. For histological analysis after specimen sacrifice, tissues were fixed in Bouin solution during 24 h and then subjected to a dehydration process with successive passages of 15 min each in increasing alcohol concentrations (70%, 96%, 90%, 100% and Xilol) to be finally embedded in paraffin blocks for staining. The histological slices were 5-micron-thick and dewaxed with Xilol for 15 min, to be later rehydrated by successive passages of 10 min each in decreasing alcohol concentrations (100%, 96%, 90%, 70%). For staining it was used the Hematoxylin and eosin technique.

### c) Western Blot

After thyroid extraction by removing lower jaw and a small part of the hyoid bone, samples were homogenized by sonication under refrigeration in lysis buffer (Tris 1,514 g, SDS 6 g and 2 beta mercaptoethanol 5 ml for 200 ml, pH 6.8) and Protease Inhibitor Cocktail (Pierce Biotechnology Inc., Massachusetts, USA) in a ratio of 10 µL per 1 ml of tissue. The supernatant solution was placed at 100°C (boiled in water) for 5 min and then centrifuged at 1600 rpm. The sample protein quantity was measured with Bradford method. Then it was carried out an SDS-PAGE in 12% polyacrylamide gel under denaturing conditions, in an electrophoresis cell (BioRad Mini Protean 3 Cell) for 90 min at 120 volts with transfer buffer (Tris 25mM; glycine 0.2 M; SDS 0.1% and pH 8.3). The loading volume per each sample was 10 to 20 µl. The volume

was decided in relation with the protein quantity present in each sample. Each sample was diluted in loading buffer in a ratio of 1:2 (Tris-HCl 0.065 M, SDS 3%, bromophenol blue 0.1%,  $\beta$ -mercaptoethanol 5% and 10% glycerol, pH 6.8). Beta actin was used as loading control. Afterwards humid electro transference and immunoblotting were performed. For the electroblotting (electrotransfer) a Polyvinylidene difluoride (PVDF) membrane (Amersham, UK) and a cell with transfer buffer were used (25 mM Tris, 192 mM glycine and 20% methanol) for 1 h at 100 mv. Subsequently, three washes were carried out of 5 min each with TBS 1X (TrisHCl 20 mM, NaCl 150 mM pH 7.8) and it was blocked with a TBS solution with Tween-20 0.2% (TBS-T) and 5% p/v of skim milk (Svelty) for 1 h at room temperature (shaking). The membrane was incubated with rabbit polyclonal primary Anti COOH-terminus NIS antibody (Millipore Corp. USA, CAT #ABC1453) at a 1:500 dilution overnight at 4°C and subsequent three washes with TBS-T and one with TBS of 5 min each. After blocking the membrane during 30 to 40 min and subsequent washes with TBS-T of 5 min each, it was incubated for 1 h at room temperature (shaking) with secondary antibody conjugated with peroxidase 1:1.500 of mouse anti-rabbit polyclonal. Finally, in order to perform detection by chemiluminescence three washes with TBS-T and one with TBS of 7 min each were carried out. After the last wash the membrane was incubated for 5 min with the reagent for enhanced chemiluminescence (ECL) (Biorad, cat #170-5060 USA) and it was exposed to X-ray plate (Kodak and GE) during 1 to 5 min and further plate development in darkroom. The developed signal was quantified with software for image analysis Scion Image Version beta 4.0.2.

#### d) Water analysis

A groundwater sampling from wells of different depth (30 to 60 meter-depth) located in the studied area (Pampeano aquifer) was carried out. The different types of analyzed water (groundwater and filtered drinking water) used in the experiments were storage and transported, sealed and refrigerated to the place where the experiments were performed in new plastic bottles of mineral water emptied and later rinsed with the collected water. Then, the bottles were filled up to the total capacity, no air gap between the lid and the content, and transported refrigerated to the place of processing. All water samples underwent a microbiologic and physiochemical analysis at the National Institute of Industrial Technology (INTI-Parque Tecnológico Miguelete, Argentina).

To determine the presence of nitrates it was used ion chromatography technique by Metrohm's 881 Compact, column Metrosep A 150/4mm, with carbonate/bicarbonate eluent, chemical suppression with conductivity detection, and calibration by peak area. For other analyzed parameter APHA-methods 2340 were

used according to the standard analysis of water for human consumption (Standard Methods for the Examination of Water and Wastewater. 1995). Reference values for water for human consumption are those of the Argentine Food Regulations. The term Undetectable was used for concentrations below the detection limit (DL) of the method of analysis.

#### e) Statistical analysis

Parametric one-way ANOVA tests were performed for the statistical analysis regarding morphology, histology and biomolecular parameters. The normal distribution was verified by means of Kolmogorov-Smirnov and Bartlett tests, and Tukey and Bonferroni post tests were carried out for the analysis of differences. A non-parametric ANOVA with Kruskal-Wallis and Dunn tests were performed for those small and asymmetric samples. For the qualitative variable analysis reflected in the contingency tables as a percentage, it was used as statistical test the Exact Fisher Test and the Katz's numerical approximation to evaluate the relative risk. In all cases it was considered as significant  $p < 0.05$  with 95% confidence intervals (CI), and in each case it was determined the interval, the average, the standard deviation; and for the percentage analysis it was used the relative risk. For that purpose, statistical GraphPad Software (Inc. San Diego, California USA, [www.graphpad.com](http://www.graphpad.com)) and Infostat (statistical program, digital version 2015. [www.infostat.com](http://www.infostat.com).) were used.

### III. RESULTS

#### a) Physicochemical analysis results of studied area water

Regarding the nitrate analysis, Sample N°1 (from 60-meter-depth wells) showed a value of 24 mg/l, exceeding EPA regulations as safety limit for water for human consumption (EPA, 2016) and Sample N°2 (from 30-meter-depth wells) showed a concentration of 83 mg/l exceeding the maximum limit of safety for water for human consumption (CAA, 2021) established in the Argentine Food Regulations (Table 1). These results should be analyzed in connection with the rest of the determinations, as nitrates may be acting *per se* or in synergy with other components, generating the thyroid disruptive effect observed in the population of those areas.

The samples from well groundwater (1 and 2) showed a higher conductivity (Table 1) which indicates an increase in salinity, probably generated by the presence of septic tanks in the vicinity (between 1 and 3 meters) of sampling areas and for the possible existence of industries near the studied area which dump liquids such as cleaning water. On the other hand, the total hardness of Sample N°3, being over 330 mg/l, is highly superior to the others, so those may be considered hard waters. This fact could favor the



reactivity of substances with possible disruptive action (De Groef et al. 2006).

High levels of arsenic, above safety level for human health by the Argentine Food Regulations (up to 0.01 mg/l), were observed in the samples of groundwater as well as in purified bottled water drunk by the population living in the area of the study (Table 2).

#### b) Morphological changes

##### i. Total time of metamorphosis. Weight and Height

Regarding the number of animals which completed metamorphosis in all before mentioned three groups, a very significant difference was observed among the groups. Larvae immerse in filtered drinking water (C) completed metamorphosis in a 100% but just a 38% for those animals in the group exposed to groundwater (E), and none in the positive control group (PC) (filtered drinking water with  $\text{KLCIO}_4$ ) ( $p < 0.001$ ). Larvae growth delay was particularly observed in the transition from stage **58NF** to **60NF** ( $p < 0.01$ ). This delay was too evident and progressive in group PC from stage **54NF** ( $p < 0.0001$ ), with a 95% confidence interval (CI) (0.6368 - 0.7923) and a relative risk that tends to infinity for the relation C Vs E. And for E vs PC the CI was 95% (0.2077 - 0.3632).

Total time of metamorphosis in group C was  $56 \pm 1.95$  days, and  $67 \pm 2.01$  days ( $p < 0.01$ ) in group E. In PC this time could not be determined due to the fact that larvae reached metamorphosis stage **62NF** but none of them reached stage **66NF**, time when the metamorphosis process is completed. This happened due to the addition of a constant dose of potassium perchlorate (0,007 mg/ml), which caused a total stop of metamorphosis at stage **62NF** (Fig. 1). Mortality per group was 10% in group E larvae exclusively ( $p < 0.001$ ).

The stage affected was prometamorphosis which is controlled by  $T_4$ . It was observed a slower larval growth in group E represented by a delay in the transition from one stage to the following during the prometamorphosis process. This difference was significant in groups E and PC vs group C during transition from stage **58NF** to **60NF** and it was noticed a significant larval growth delay in group E Vs C during the transition from stage **54NF** to **60NF** ( $p < 0.01$ ) with 0.69 relative risk (RR). The delay was even more pronounced in PC group larvae vs C, ( $p < 0.002$ ), with a 0.46 RR. The weight at stage **58NF** was significantly lower in group E Vs C and PC ( $p < 0.05$ ), with a 95% confidence interval (CI) (54.157 - 364.69) (Fig. 2). It was noticed a significant difference in height, but this difference was smaller in group E and PC vs C ( $p < 0.05$ ), with a 95% confidence interval (CI) (8.667 - 5.448: E vs C, -6.750 - 4.619: PC vs C). No significant difference was observed between groups C and PC (Fig. 2).

From the above analyzed we conclude that the delay occurs in the transition from stage **54NF** to **60NF** in group E and PC vs C, with a larval growth delay in

groups E and PC. In group E this delay is evidenced by a decrease in the final size of animals, which achieved less weight and height than the ones in the other two groups (C and PC) (Picture 1).

#### c) Histological changes

##### i. Follicular colloidal area

During **58NF** stage metamorphosis it was observed an increase in the colloid area size in group E Vs C and PC ( $p < 0.05$ ) with a 95% CI (E vs C: -9184.7 - 179.62 and E vs PC: 2252.2 - 9253) (Fig. 3) ( $p < 0.001$ ). On the other hand, the colloidal area was smaller in group C vs PC ( $p > 0.001$ ) with a 95% CI (-2336.2 - 4.477) (Pic.2).

##### ii. Glandular hyperplasia degree

Thyroid gland hyperplasia during stage **58NF** was degree 1 in group C, degree 2 in groups E and PC. The differences observed in group C Vs E were highly significant ( $p < 0.0001$ ), with a 95% CI (0.3680 - 0.6860) and 0.33 RR. There were also significant differences between groups C and PC ( $p < 0.002$ ) with 95% CI (0.2708 - 0.4799). No significant differences were observed between groups E and PC (Fig. 4).

##### iii. Number of filled follicles

The number of filled and empty follicles per gland was also significantly different being the number of filled follicles higher in group E Vs C and PC ( $p < 0.02$ ) with 95% CI (0.4906 - 0.8303) and 0.66 RR. Nevertheless, there were no significant differences between C and PC groups (Fig. 5).

##### iv. Height of follicular epithelium

The height of the follicular epithelium showed significant differences in groups C vs E ( $p < 0.0001$ ) with a 95% IC (-181.22 - 96.084) and in groups E Vs PC ( $p < 0.0001$ ) with a 95% CI (130.56-202.21). In increasing order, the height was less in group PC than in C and in group C than in E (Pic. 3). The average height of the follicular epithelium for each group was: C:  $166.23 \pm 43.23$ ; E:  $284.02 \pm 68.12$ ; PC:  $128.64 \pm 35.69$  um (Fig. 6).

#### d) Molecular changes

##### i. NIS expression analysis

When data logarithmic correction was made it showed that NIS protein expression increases in groups E and PC being this a significant difference ( $p < 0.05$ ) (Fig. 7). The average in increasing order was  $E > PC > C$ . Registered values measured in optical density were: C:  $680.7 \pm 196.92$ , E:  $1251.02 \pm 702.94$ , PC:  $1059 \pm 592.85$  (Fig. 6).

## IV. DISCUSSION

Endemic regions of hypothyroidism and goiter without iodine deficiency have been described, suggesting the consumption of EDs by the population living in these areas as a predisposing factor. (Blount

BC. et al. 2006). Our study, carried out in the southern suburbs of Buenos Aires, found levels of nitrates and arsenic in the groundwater above the safety limit for human consumption. Furthermore, in studies made in nearby rivers, other pollutants with endocrine disruptive action were detected, among them: lead, chrome, hydrocarbon and polychlorinated biphenyls (PCBs) (Janiot L. 2000). These elements may contaminate the groundwater layers, specially the superficial ones by runoff from water tables in low-gradient streams. In the area of study, the water to drink or to irrigate is obtained from these water layers, being the Pampeano and Puelche aquifers the sources from where most of the population gets their water. The superficial Pampeano aquifer is free and often contaminates the deep Puelche aquifer which is semi confined. The last one represents one of the drinking water reservoirs most important of Argentina and Latin America (Adema MP. 2017 and Ingeniería Geotécnica y Ambiental. 2005).

The action of thyroid endocrine disruptors (TEDs) may alter the synthesis, storage, transport and catabolism of hormone homeostasis (Colborn T. et al 1993) and may decrease the production of thyroid hormones (Kleiman DL. et al. 1989) by acting on membrane transporters such as NIS. In the case of nitrates, the inhibition of the sodium-iodide symporter (NIS) interferes with iodine uptake at thyrocyte level, first step in thyroid hormone synthesis (De Groef B. et al 2006). This leads to hypothyroxinemia with the following increase of TSH (Manzon RG. et al. 2004), which induces cell proliferation as an adaptive response. Thus, generating an increase in gland size and changes in gland histoarchitecture, which in humans may cause goiter development (Brauer VF. et al. 2006).

The decrease in thyroid hormone levels affects human development, as well as it does in larvae metamorphosis. In this study we have shown a delay in the development, and changes in the body morphology during larvae metamorphosis of *Xenopus laevis* exposed to contaminated water, causing caused by thyroid disruption. We have demonstrated a longer period of prometamorphosis and smaller weight and height of specimens at stage 58NF. These differences could be explained by the need to reach a metabolic threshold, which allows them to complete the morphological changes of this stage. This could be determined by the acquisition of an adequate level of thyroid hormones. In case that this does not happen, it can cause a stop in larval development. This fact has been observed in other amphibians and urodele, which develops a state called *neoteny* (Galton VA. 1992), determined by a complete brake on the metamorphic process in adverse environmental situations. This fact is similar to the one observed in our experimental Positive Control (PC) group, in which larvae were exposed to a constant dose (0.007 mg/l) of potassium perchlorate (known as a NIS inhibitor), suffering a complete stop of its metamorphic

development; therefore, no larvae completed the metamorphosis process.

The observed differences could be explained by the negative feed-back made by the  $T_3$  and  $T_4$  at tadpole hypophysis level which is operational at prometamorphosis onset. Thyroid hormones may negatively regulate the mRNA expression for the TSH synthesis during metamorphosis. The mRNA expression for the thyroid hormone receptor increases during the larval development throughout prometamorphosis and peaks at climax (Opitz R. et al. 2006). The presence in water of NIS inhibitors, such as nitrates, could be interfering with the proper production of thyroid hormones in larvae.

We have known for years that *Xenopus laevis* are extremely sensitive to water soluble substances as nitrates and perchlorates, even in low concentration, due to their aquatic life. For this reason, *Xenopus laevis* was chosen as experimental model in this work (Kloas W. 2002).

Histologically, the follicles constitute the anatomical functional unit of the thyroid gland in amphibians and in humans. Its follicular epithelium and the colloid constituted by thyroglobulin change their histological appearance depending on the secretory phase. These events may be altered by thyroid disruptors, being the histologic changes a sensitive parameter to determine the level of action of this disruptor (Wolff J. 1998). Our analysis showed a change in gland histoarchitecture like hyperplasia and hypertrophy of the follicular epithelium and an increase of the colloid volume in the thyroid gland follicles in prometamorphic larvae.

The thyrocyte uptakes iodine against gradient by the sodium-iodide symporter (NIS) located at the basement membrane, with energy expenditure. This transporter is inhibited by nitrates and other disruptors. The NIS expression is stimulated by the TSH, which involves the regulation of transcription factor as TTF1, TTF2 and PAX8 (Rivolta CM. et al. 2005).

The inhibitory action of thyroid disruptors on the NIS co-transporter and the changes on the metamorphosis (Furlow JD. et al. 2006 and Degitz S. et al 2006), as well as histological and biomolecular thyroid changes, have been assessed (Hood A. et al. 1999; Below H. et al 2008 and Mukhopadhyay S. et al. 2005). The increase in the NIS protein expression level noticed during larvae prometamorphosis exposed to nitrate contaminated water, could be the result of an adaptive mechanism trying to compensate its functional state.

Differences observed in our study in larval morphology as well as in glandular histoarchitecture during the different stages of *Xenopus laevis* metamorphosis between E and C groups may be explained by the presence of one or more substances with a thyroid disruptive action in groundwater of the studied area. These substances could be interacting in



a synergetic way on more than one level on the thyroid gland. This could explain what happens with arsenic. The arsenic, as the nitrates, was detected in concentrations considered as unfit for human consumption by the Argentine Food Regulations. The chronic exposure to an excess of arsenic in drinking water has been strongly linked to higher risk in humans. Arsenic has been shown to be a powerful endocrine disruptor in low levels, changing the genic regulation mediated by thyroid receptors (Davey JC. 2008). The synergistic action of nitrates and arsenic could explain the mortality observed exclusively in this group.

## V. CONCLUSION

The nitrates present in groundwater, as well as other possible endocrine disruptors such as arsenic, produce morphological alteration in the *Xenopus laevis* tadpoles, as well as histological and molecular thyroid changes when exposed to this type of water during their metamorphosis. These events are related to an increase of the NIS expression levels during prometamorphosis stage. Despite this adaptive change, it is not possible to compensate for the thyroid alteration generated by nitrates, thus not achieving the morphological changes necessary to adequately complete this stage. New studies must be carried out to better understand the mechanisms that lead to these alterations.

### Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Table 1:** Physicochemical analysis of groundwater (Pampean Aquifer, Buenos Aires, Argentina) sampling

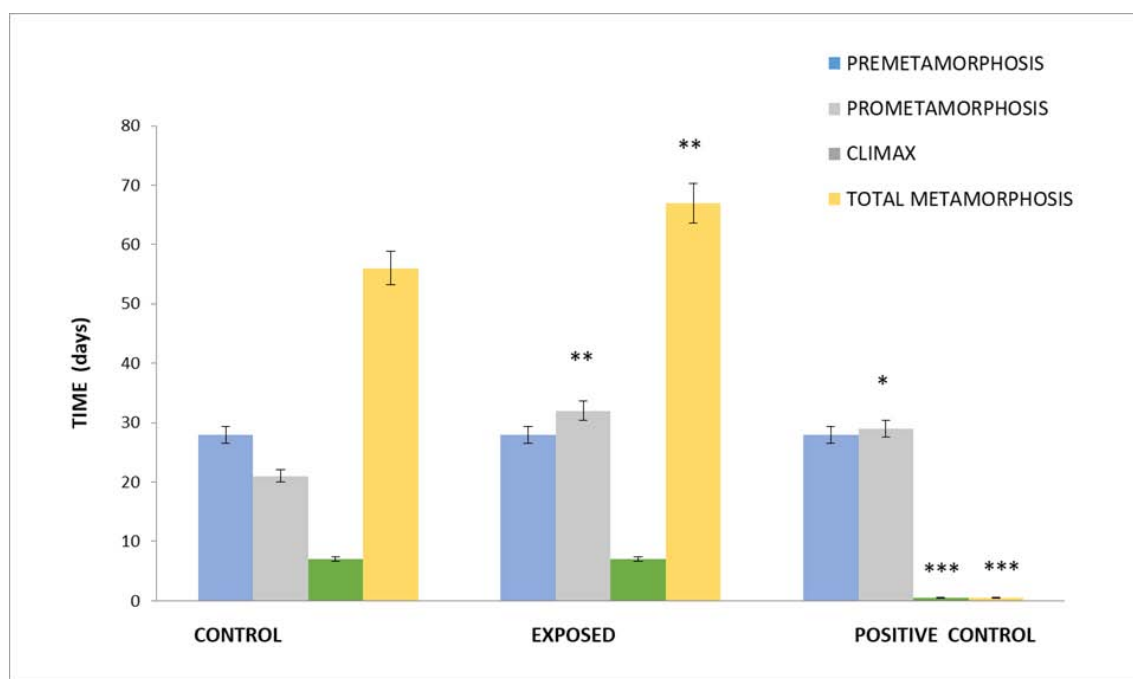
<b>SAMPLE 1</b>  <b>Well depth: 60 meters</b>	COLOUR	Greenish <sup>(a)</sup>
	ODOR	Odorless
	SEDIMENT	Null
	pH	7.5
	RESIDUAL ACTIVE CHLORINE	0,00 ppm
	CONDUCTIVITY	729 micros/cm <sup>(a)</sup>
	TOTAL DISSOLVED SOLIDS (TDS)	525 mg/l
	TOTAL ALKALINITY (CO <sub>3</sub> Ca)	312 mg/l
	TOTAL HARDNESS (CO <sub>3</sub> CA)	114 mg/l
	CHLORINES (CL)	36 mg/l
	SULPHATES (SO <sub>4</sub> <sup>2-</sup> )	20 mg/l
	AMMONIA (NH <sub>4</sub> <sup>+</sup> )	Undetectable
	NITRITES (NO <sub>2</sub> ):	0.01 mg/l
	NITRATES (NO <sub>3</sub> ):	24 mg/l*
	CHROMIUM (Cr <sup>+6</sup> ):	Undetectable
<b>DRINKING WATER</b>  (Extraction of chlorine by filter)	COLOUR	Colorless
	ODOR	Odorless
	SEDIMENT	Plentiful
	pH	7.6
	RESIDUAL ACTIVE CHLORINE	0,00 ppm
	CONDUCTIVITY	685 micros/cm
	TOTAL DISSOLVED SOLIDS	493 mg/l
	TOTAL ALKALINITY (CO <sub>3</sub> Ca)	351 mg/l
	TOTAL HARDNESS (CO <sub>3</sub> CA)	80 mg/l
	SEDIMENT	18 mg/l
	SULPHATES (SO <sub>4</sub> <sup>2-</sup> )	20 mg/l
	AMMONIUM(NH <sub>4</sub> <sup>+</sup> )	Undetectable
	NITRITES (NO <sub>2</sub> ):	Undetectable
	NITRATES (NO <sub>3</sub> ):	< 5 mg/l
	CHROMIUM (Cr <sup>+6</sup> ):	Undetectable

SAMPLE 2 Well depth: 30 meters	COLOUR	Colorless
	ODOR	Odorless
	SEDIMENT	Barely detectable
	pH	7.3
	RESIDUAL ACTIVE CHLORINE	0,00 ppm
	CONDUCTIVITY	982 micros/cm <sup>(a)</sup>
	TOTAL DISSOLVED SOLIDS	707 mg/l
	TOTAL ALKALINITY (CO <sub>3</sub> Ca)	409 mg/l
	TOTAL HARDNESS (CO <sub>3</sub> CA)	336 mg/l <sup>(a)</sup>
	CHLORINES (CL)	87 mg/l
	SULPHATES (SO <sub>4</sub> <sup>2-</sup> )	25 mg/l
	AMMONIA (NH <sub>4</sub> <sup>+</sup> )	Undetectable
	NITRITES (NO <sub>2</sub> ):	0.01 mg/l
	NITRATES (NO <sub>3</sub> ):	83 mg/l *
	CHROMIUM (Cr <sup>+6</sup> ):	Undetectable
SAMPLE 3 Drinking water (can)	COLOUR	Colorless
	ODOR	Odorless
	SEDIMENT	Plentiful
	pH	7.6
	RESIDUAL ACTIVE CHLORINE	0,00 ppm
	CONDUCTIVITY	395 micros/cm <sup>(a)</sup>
	TOTAL DISSOLVED SOLIDS	284 mg/l
	TOTAL ALKALINITY (CO <sub>3</sub> Ca)	175 mg/l
	TOTAL HARDNESS (CO <sub>3</sub> CA)	112 mg/l <sup>(a)</sup>
	CHLORINES (CL)	29 mg/l
	SULPHATES (SO <sub>4</sub> <sup>2-</sup> )	20 mg/l
	AMMONIA (NH <sub>4</sub> <sup>+</sup> )	Undetectable
	NITRITES (NO <sub>2</sub> ):	Undetectable
	NITRATES (NO <sub>3</sub> ):	< 5 mg/l
	CHROMIUM (Cr <sup>+6</sup> ):	Undetectable

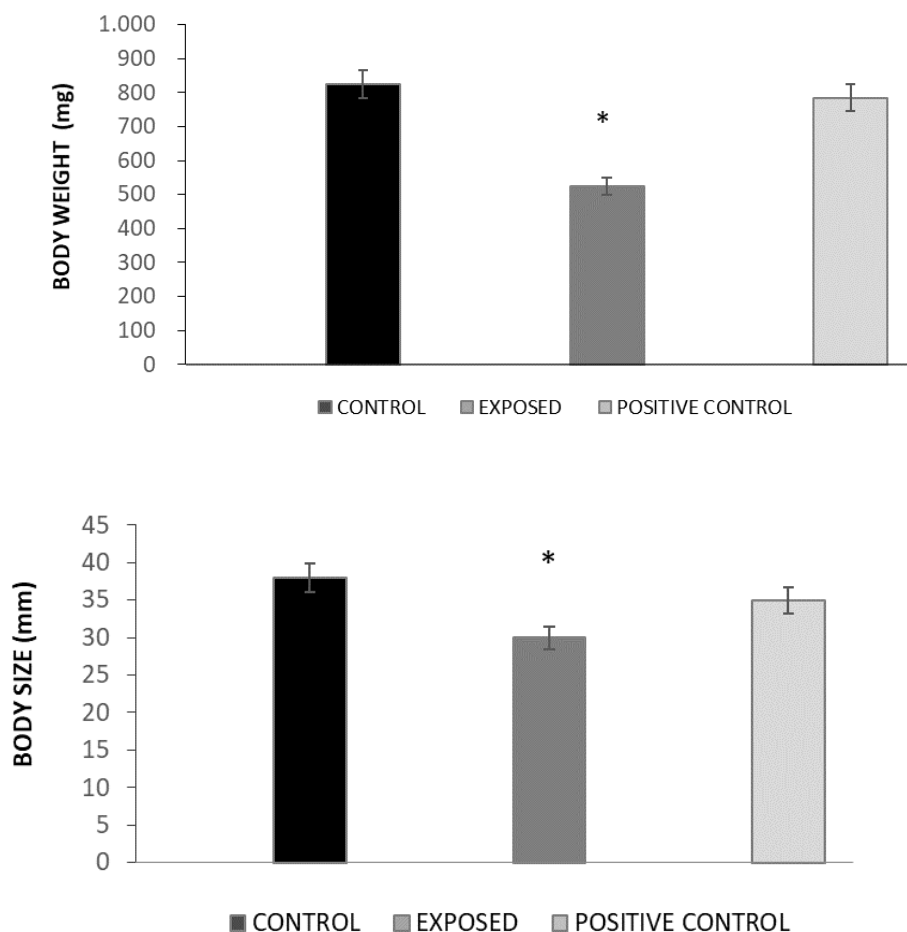
\*On or exceeding the limits for safety values. <sup>(a)</sup> Differences in values between drinking water and purified bottled water

Table 2: Levels of arsenic and others elements.

DETERMINATION TYPE	PURIFIED BOTTLED WATER	GROUNDWATER
TOTAL HARDNESS (CO <sub>3</sub> Ca)	57 ± 2.0 %	64 ± 20 %
CALCIUM	12.5 ± 2.5 %	13.5 ± 2.5 %
MAGNESIUM	6.4 ± 3.0 %	7.2 ± 3.0 %
ARSENIC	0.02-0.07 mg/l	0.05 mg/l
MAGNESIUM	< 0,05	< 0,05
SODIUM+POTASSIUM	132	183



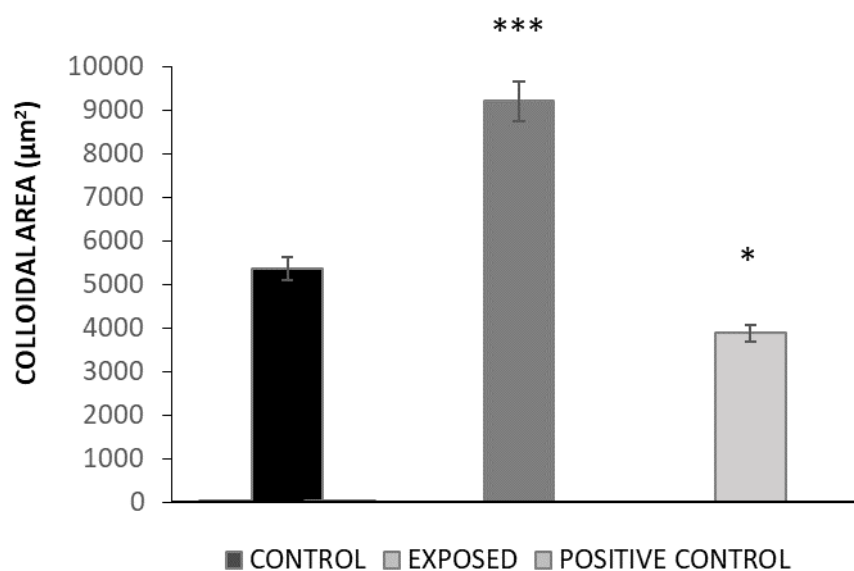
**Figure 1:** Metamorphosis periods (premetamorphosis, prometamorphosis and climax) per group and water type of *X. laevis* larvae under Control treatment: C, Exposed: E, and Positive Control: PC. \* $p < 0.05$ , \*\* $p < 0.001$ , \*\*\* $p < 0.0001$  vs. Control



**Figure 2:** *Xenopus laevis* larvae body weight and size at stage 58NF. \* $p < 0.05$  vs. Control.

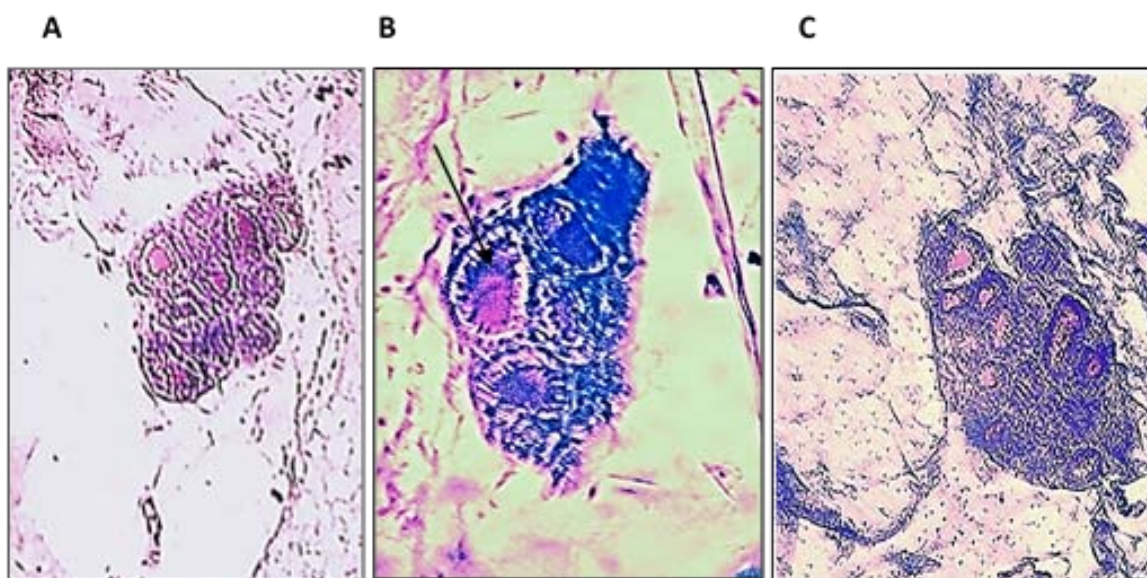


**Picture 1:** Larvae morphological development change at stage 58NF (prometamorphosis), showing a delay in the Exposed groups and Positive Control Vs Control.

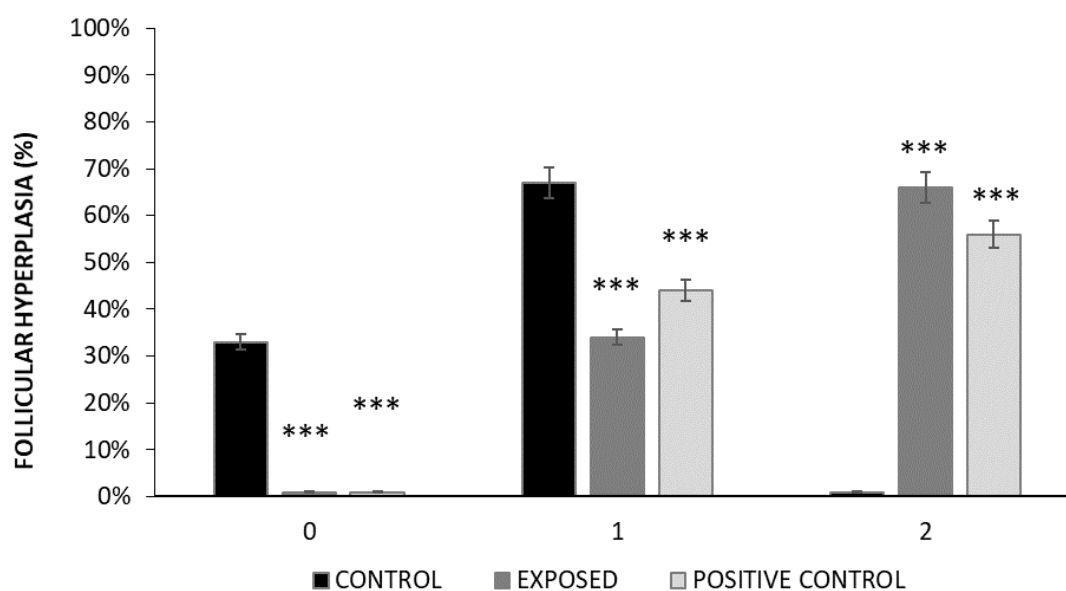


**Figure 3:** Thyroid gland colloidal area at stage 58NF. \* $p < 0.05$ , \*\*\* $p < 0.0001$  vs. Control.





**Picture 2:** Optical microscopy (10x) of the follicular colloid area in *Xenopus laevis* thyroid glands at stage **58NF**: A) Control, B) Exposed: where a bigger size gland and an increase in the colloid area can be observed, C) Positive Control.



**Figure 4:** Percentage of follicular hyperplasia degrees (0, 1, 2) in each experimental group during stage **58NF**.  
\*\*\* $p < 0.0001$  vs Control.

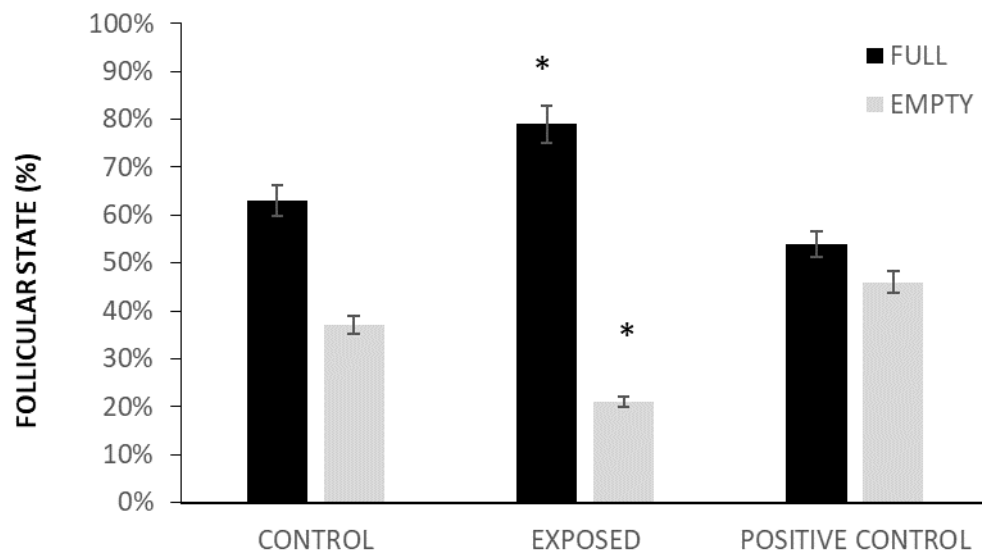
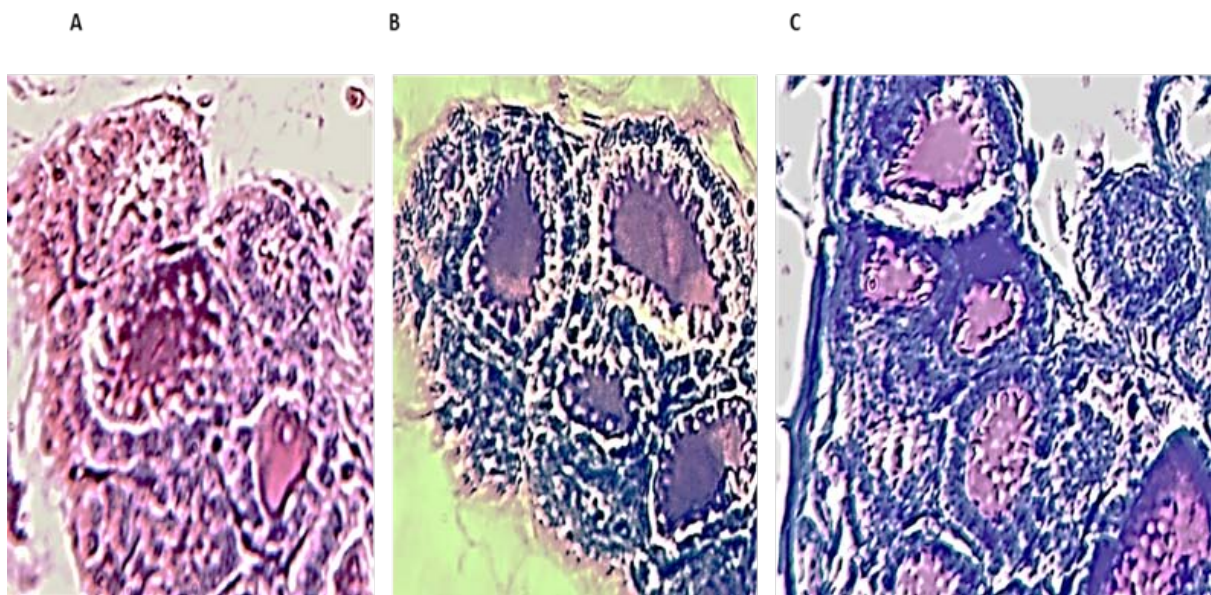


Figure 5: Percentage of filled and empty follicles during stage 58NF. \* $p < 0.01$  vs Control.



Picture 3: Optical microscopy (40x) of the thyroid gland in *Xenopus laevis* at stage 58NF showing colloid area, size and degree of follicular hyperplasia: A) Control, B) Exposed, C) Positive Control.

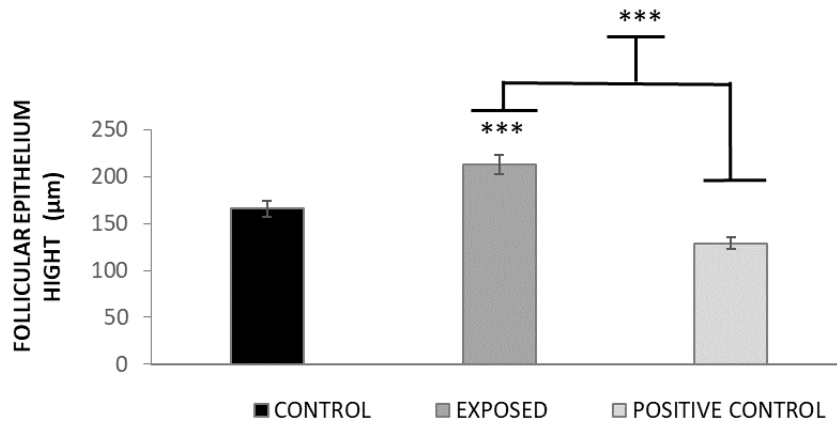


Figure 6: Follicular epithelium height at stage 58NF. \*\*\* $p < 0.0001$  vs Control and vs Positive Control.

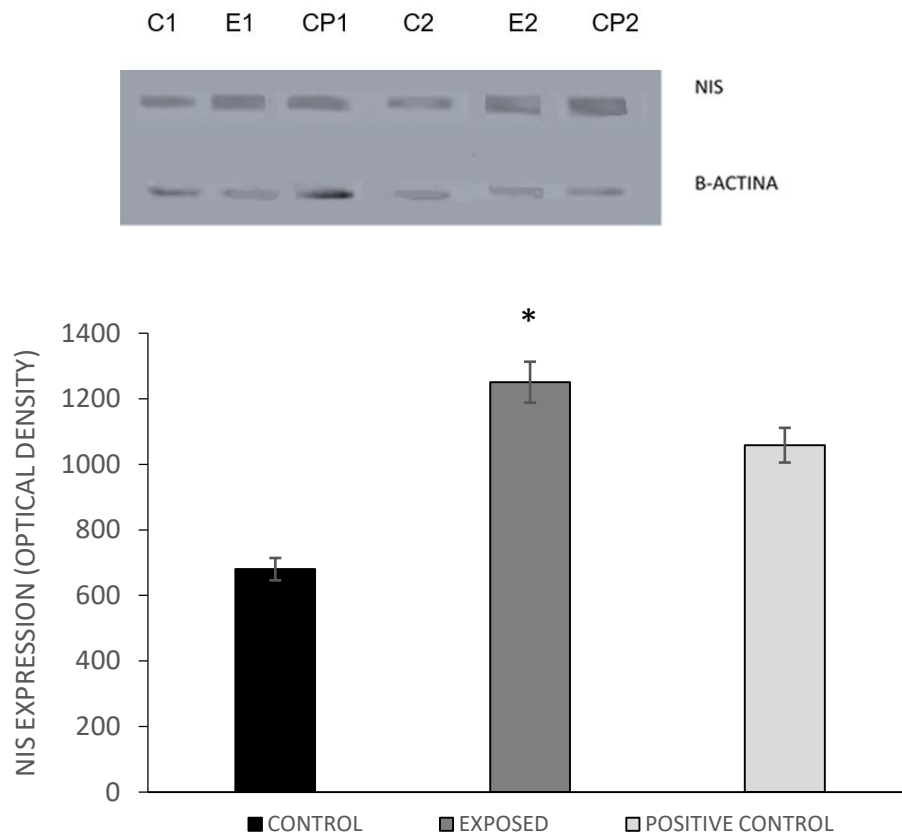


Figure 7: NIS protein expression at stage 58NF was higher in groups Exposed (E 1, 2) and Positive Control (PC 1, 2) vs Control group (C 1, 2) \* $p < 0.05$ .



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# Development of Dietary Supplement Capsules “Nigelit” using Mathematical Methods

By Kh. K. Bekchanov, S. F. Iskandarova & N. A. Djabbarov

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**Abstract-** The research featured the development of formulae and technology of obtaining biologically active additives for functional foods with potential anti-inflammatory activity using a substance containing zinc (microelement) and plant raw material as source of natural biologically active substances. The relevance of the study is comes from the need for more effective use of natural biologically active compounds, the need to expand the range of domestic dietary supplements, in particular, those recommended for the prevention of disorders of the prostate activity and erectile dysfunction, with natural ingredients and import substitution. On the basis of scientific data and the results of the conducted research, substance containing zinc and a promising plant raw material containing biologically active substances were selected; their safety and potential properties were assessed. The optimal ratio and dosage of active ingredients in the composition has been scientifically substantiated.

**Keywords:** *biologically active additives, dietary supplement, raw material, mathematical method, experimental design, factor, optimization parameters, capsules, “nigelit”, excipients.*

**GJMR-B Classification:** LCC Code: KF49



*Strictly as per the compliance and regulations of:*





# Development of Dietary Supplement Capsules “Nigelit” using Mathematical Methods

Kh. K. Bekchanov <sup>α</sup>, S. F. Iskandarova <sup>σ</sup> & N. A. Djabbarov <sup>ρ</sup>

**Abstract-** The research featured the development of formulae and technology of obtaining biologically active additives for functional foods with potential anti-inflammatory activity using a substance containing zinc (microelement) and plant raw material as source of natural biologically active substances. The relevance of the study is comes from the need for more effective use of natural biologically active compounds, the need to expand the range of domestic dietary supplements, in particular, those recommended for the prevention of disorders of the prostate activity and erectile dysfunction, with natural ingredients and import substitution. On the basis of scientific data and the results of the conducted research, substance containing zinc and a promising plant raw material containing biologically active substances were selected; their safety and potential properties were assessed. The optimal ratio and dosage of active ingredients in the composition has been scientifically substantiated. The technological properties of the mixtures of active ingredients that determine the choice of technology and the quality of the finished product were studied. Researches were carried out to select the optimal composition for the capsules “Nigelit” using a mathematical method of experimental design, the  $4 \times 4$  Latin square. The experimental results were processed using mathematical methods of statistical and variance analysis, involving the Fisher statistic - Fstatistic (also called the F-ratio) and the generalized desirability function. As a result, the composition and technology of dietary supplement - capsules “Nigelit” were developed.

**Keywords:** biologically active additives, dietary supplement, raw material, mathematical method, experimental design, factor, optimization parameters, capsules, “nigelit”, excipients.

## 1. INTRODUCTION

Erectile dysfunction or the inability to achieve or maintain an erection sufficient for satisfying sexual activity, often becomes a real problem for men. According to the WHO information every 10th managed older 21 has the erectile disorder and after the age of 60 years every third man is not able to perform sexual

intercourse. About 35% of men aged 40 to 70 years suffering from partial or total inability to achieve erection [1].

To date, effective approaches of pharmacological correction of disorders of sexual function in men have been developed. Depending on the indications for use, these drugs should have separately or in combination neurotropic, vasodilating, anti-inflammatory, antimicrobial, antihypoxic, immunotropic effect [1]. The basis of drug therapy for erectile dysfunction, as a rule, is made up of phosphodiesterase inhibitors: vardenafil, sildenafil, tadalafil [1]. However, all of these drugs are available with a doctor's prescription. At the same time, significant advances in the prevention and treatment of male genital pathology can be achieved with the use of over-the-counter herbal remedies. Compared to synthetic ones, natural components have a milder and more versatile effect due to the variety of components actively affecting the body and, as a rule, rarely cause side effects [1]. Most of the herbal remedies for correcting erectile dysfunction are presented in the form of biologically active additives.

Today the market of dietary supplements for the correction of erectile dysfunction mainly offers complex drugs of a certain functional orientation, most often combining herbal ingredients with a pronounced stimulating effect on sexual function and a general tonic effect [1].

When reviewing the literature, it was found that one of the types of plant raw materials that have a beneficial effect on the function of the prostate is the seeds of *Nigella sativa* [2], and of the microelement – zinc [3, 4].

Based on the analysis of the pharmaceutical market of the manufactured dosage forms, we have selected hard gelatin capsules as the rational dosage form for the medicinal preparation under development. Capsules represent a prospective solid dosage form with a number of advantages. For instance, they are attractive in appearance, easy to swallow, contain accurate dose, protect encapsulated medications against light, air and moisture since capsule shells provide a high level of airtightness, can quickly swell up, dissolve and get absorbed in the gastrointestinal tract, have high bioavailability [5].

The aim of the research is to develop the composition and technology for obtaining dietary

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supplements with specified characteristics using plant raw materials - seeds of *Nigella sativa* and a microelement – zinc.

## II. MATERIALS AND METHODS

Seeds of *Nigella sativa*, zinc sulphate, their mixture, their mixtures with different excipients and capsules on their base were selected as the materials of research. The technological properties of the materials that determine the composition, technology and the quality of the finished product and the capsule disintegration were studied using conventional methods, described in [6, 7, 8]. In order to select the optimal composition for the capsules "Nigelit" a mathematical method of experimental design, the  $4 \times 4$  Latin square was used. The experimental results were processed using mathematical methods of statistical and variance analysis, involving the Fisher statistic (F statistic) and the generalized desirability function [9, 10, 11, 12]. In order to create granules moisture activated dry granulation (MADG) was used [13, 14, 15].

## III. RESULTS AND DISCUSSION

For the development of the dietary supplement, firstly, a suitable dose of the microelement – zinc and plant raw material containing biologically active substances were calculated, based on the study of their safety and potential properties. Then the technological properties of the mixture of active components were studied. It was established that the mixture of active components has unsatisfactory technological properties in particular flow ability. This makes obtaining capsules of an acceptable quality from such a material impossible. In order to eliminate these disadvantages, and to produce good-quality capsules, it is necessary to incorporate excipients, and to use granulation, which prevents the segregation (stratification) of the mixture [16].

In order to choose the most rational composition and the encapsulation technology for the capsules "Nigelit", we took advantage of a mathematical method of experimental design, the method of the  $4 \times 4$  Latin square and performed analysis of variance. The use of this method makes it possible to significantly reduce the experimental error and to quantify the effect of various factors on the optimization parameters [9, 10, 11, 12]. In this case, flowability, bulk density, angle of repose of the granular materials and disintegration time of capsules were selected as the optimization parameters (see table 1); while fillers, antiadherents (anti-sticking agents), disintegrants and glidants were selected as factors affecting optimization parameters (see table 2). Technological properties of the granular materials and the capsule disintegration were studied using conventional methods [6, 7, 8]. In order to create granules moisture activated dry granulation (MADG) was used [13, 14]. This method allows for simultaneous mixing and of ingredients, as well as forming of a product with homogeneous dispersion [13]; minimization grinding of such issues with wet granulation as the need for a significant amount of granulating liquid, the duration and energy intensity of the process of mixing the wet mass, sensitivity of individual components of the mixture to high moisture levels, the necessity of using granule-forming *apparatus* in most cases, the long-lasting stage of drying the granules, accompanied by the unfavorable effect of temperature on active pharmaceutical ingredients, as well as the cumbersome equipment required for the air preparation and air purification processes [15]. The main advantage of this granulation method is that the resulting granules do not need to be dried - this speeds up the technological process, reduces labor and energy costs. Moreover, the resulting granules do not need additional grinding due to the characteristic homogeneous particle-size distribution [13]. Purified water was used as the moisturizing liquid for granulation.

Table 1: Optimization parameters for the capsules "Nigelit"

Optimization parameters (Y)			
Y <sub>1</sub>	Y <sub>2</sub>	Y <sub>3</sub>	Y <sub>4</sub>
Flowability (10 <sup>-3</sup> kg/s)	Bulk density (kg/m <sup>3</sup> )	Disintegration (min.)	Angle of repose (°)

Table 2: Factors affecting the optimization parameters of capsules "Nigelit"

Name of the capsules	Factors			
	A - fillers	B - antiadherents	C - disintegrants	D - glidants
	a <sub>1</sub> - starch	b <sub>1</sub> - magnesium stearate	c <sub>1</sub> - CMC	d <sub>1</sub> - starch



"Nigelit"	$a_2$ - Primalac 60	$b_2$ – calcium stearate	$c_2$ - MCC Arbocel A 300	$d_2$ – aerosil
	$a_3$ - MCC Arbocel A 300	$b_3$ - stearic acid	$c_3$ - UAP	$d_3$ – talc
	$a_4$ – dextrin	$b_4$ – kaolin	$c_4$ -Na-CMC	$d_4$ – PEG-400

In order to select the optimal composition for capsules "Nigelit" various mixtures of active components and excipients were designed and prepared according to the formulations presented in table 3. Each mixture prepared according to the formulations was granulated

using moisture activated dry granulation method. Then the technological properties of these mixtures and the disintegration of the capsules prepared on their base were studied (see table 3).

**Table 3:** Experiment design matrix and research results on the effect of the excipients on the optimization parameters for capsules "Nigelit"

(Symmetric fractional factorial experiment, 16 out of 256, 1/16 fraction)

Experiment number	Factors				Optimization parameters				$D$
	A	B	C	D	$Y_1$ , $10^{-3}$ kg/s	$Y_2$ , kg/m <sup>3</sup>	$Y_3$ , min.	$Y_4$ , °	
1	$a_1$	$b_1$	$c_1$	$d_1$	4,5	387	30	40	0,47
2	$a_1$	$b_2$	$c_2$	$d_2$	4,3	386	32	43	0,44
3	$a_1$	$b_3$	$c_3$	$d_3$	5,0	400	33	42	0,48
4	$a_1$	$b_4$	$c_4$	$d_4$	4,1	385	38	46	0,33
5	$a_2$	$b_1$	$c_1$	$d_2$	4,1	380	32	41	0,42
6	$a_2$	$b_2$	$c_2$	$d_1$	4,3	381	28	41	0,47
7	$a_2$	$b_3$	$c_3$	$d_3$	4,8	392	35	42	0,43
8	$a_2$	$b_4$	$c_4$	$d_4$	3,9	380	40	46	0,29
9	$a_3$	$b_1$	$c_2$	$d_3$	5,5	390	20	38	0,69
10	$a_3$	$b_2$	$c_1$	$d_2$	4,8	377	25	43	0,54
11	$a_3$	$b_3$	$c_3$	$d_1$	5,1	380	28	44	0,52
12	$a_3$	$b_4$	$c_4$	$d_4$	4,7	378	30	46	0,46
13	$a_4$	$b_1$	$c_1$	$d_4$	4,4	388	27	43	0,51
14	$a_4$	$b_2$	$c_2$	$d_3$	5,2	400	25	40	0,61
15	$a_4$	$b_3$	$c_3$	$d_2$	4,5	386	30	46	0,45
16	$a_4$	$b_4$	$c_4$	$d_1$	4,8	390	33	43	0,46

Then the results of studying the optimization parameters (table 3) were subjected to analysis of variance which allows us to test the null hypothesis (all means are equal) against the alternative hypothesis (at least one mean is different) with a specified value of alpha (in our case the value of alpha ( $\alpha$ ) or the level of significance is equal to 0.05 or 5%) and probability (in

our case the value of probability (P) is equal to 0.95 or 95%). In other words analysis of variance allows us to determine the significance of the studied factors A, B, C, D for the optimized parameters  $Y_1$ ,  $Y_2$ ,  $Y_3$ ,  $Y_4$  (table 4) at a given level of significance [11, 17].

It should be mentioned that if the F test statistic (observed) is greater than the F critical value, i.e.  $F_{\text{statistic}} >$

$F_{critical}$ , then the hypothesis of similarity or the null hypothesis  $H_0$  is rejected, and the hypothesis of the difference or the alternative hypothesis  $H_1$  is accepted with a level of significance  $\alpha = 0.05$  or 5%. This means that the factor significantly affects the change in the output data - the values of the optimization parameter and the data depend on the factor with a probability of  $P = 0.95$  ( $P = 1 - \alpha$ ) or  $P = 95\%$  ( $P = 100 - \alpha$ ). If the F test

statistic (observed) is less than the F critical value, i.e.  $F_{statistic} < F_{critical}$ , then the hypothesis of similarity or null hypothesis  $H_0$  is accepted, and the hypothesis of difference or alternative hypothesis  $H_1$  is rejected with a level of significance  $\alpha = 0.05$  or 5%. This means that the factor does not significantly affect the output data - the values of the optimization parameter with a probability of  $P = 0.95$  ( $P = 1 - \alpha$ ) or  $P = 95\%$  ( $P = 100 - \alpha$ ).

**Table 4:** Analysis of variance of the experimental data from the study of the indicators of the capsules "Nigelit"\*

(Four-factor analysis of variance without replication)

Optimization parameters	Source of variance	Degrees of freedom (df)	Sum of squares (SS)	Mean square (MS)	$F_{statistic}$	$F_{critical}$
$Y_1$ –Flowability, $10^{-3}$ kg/s	Factor A	3	1.26	0.42	3.02	3.49
	Factor B	3	0.45	0.15	0.74	3.49
	Factor C	3	0.73	0.24	1.34	3.49
	Factor D	3	1.66	0.55	5.23	3.49
$Y_2$ –Bulk density, kg/m <sup>3</sup>	Factor A	3	268.5	89.5	2.26	3.49
	Factor B	3	78.5	26.17	0.47	3.49
	Factor C	3	156.5	52.17	1.07	3.49
	Factor D	3	467.5	155.83	6.79	3.49
$Y_3$ – Disintegration, min.	Factor A	3	174.75	58.25	3.41	3.49
	Factor B	3	172.25	57.42	3.32	3.49
	Factor C	3	182.25	60.75	3.69	3.49
	Factor D	3	66.75	22.25	0.85	3.49
$Y_4$ - Angle of repose, °	Factor A	3	0.5	0.17	0.02	3.49
	Factor B	3	51.5	17.17	5.49	3.49
	Factor C	3	51.5	17.17	5.49	3.49
	Factor D	3	48.5	16.17	4.79	3.49

\*Analysis of variance was conducted on the experimental data from table 3, and the statistical indicators in table 4 were calculated, using ANOVA module of the statistics software MiniTab.

The results of the analysis of variance (table 4) allow us to state the following:

- The selected types of fillers (Factor A) does not have a significant effect on the flowability ( $Y_1$ ), bulk density ( $Y_2$ ), angle of repose ( $Y_4$ ) of the granular materials as well as the disintegration of capsules ( $Y_3$ );
- The selected types of antiadherents (Factor B) has a significant effect on the angle of repose ( $Y_4$ ) of the granular materials and does not have a significant effect on the flow ability ( $Y_1$ ), bulk density ( $Y_2$ ) of the granular materials as well as the disintegration of capsules ( $Y_3$ );
- The selected types of disintegrants (Factor C) has a significant effect on the disintegration of capsules ( $Y_3$ ) and the angle of repose ( $Y_4$ ) of the granular materials and does not have a significant effect on the flow ability ( $Y_1$ ), bulk density ( $Y_2$ );
- The selected types of glidants (Factor D) has a significant effect on the flow ability ( $Y_1$ ), bulk density ( $Y_2$ ), angle of repose ( $Y_4$ ) of the granular materials and does not have a significant effect on the disintegration of capsules ( $Y_3$ ).

The overall (generalized) evaluation of the optimization parameters - the disintegration of capsules and the technological properties of the granular materials (model mixtures)- was carried out using a desirability function [11, 18]. In order to generalize the values of the optimization parameters that have different units of measurement, we used the well-known and

widely accepted Harrington's desirability function, first introduced by him in solving quality control problems of mass production. The Harrington's scale establishes a correspondence between linguistic evaluations of desirability of the values of the indicator  $x$  and the numerical intervals  $d(x)$  (table 5) [11, 19].

Table 5: Numerical intervals of the Harrington's scale

Linguistic evaluation	Intervals of the desirability function values $d(x)$
Very good	1.00 - 0.80
Good	0.80 - 0.63
Satisfactory	0.63 - 0.37
Bad	0.37 - 0.20
Very bad	0.20 - 0.00

In order to construct the desirability function scale of the optimization parameters for the capsules "Nigelit" (Fig. 1), the method of quantitative analysis was used with the range of desirability values between 0 and 1 (Table 5). The value  $d = 1$  corresponds to the best value of the optimization parameters, while  $d = 0$  - to their worst value of ones. The intermediate values of the desirability function reflect specific levels of the product quality: very bad (0.00 - 0.20), bad (0.20 - 0.37), satisfactory (0.37 - 0.63), good (0.63 - 0.80) and very good (0.80 - 1.00). Conversion of the natural values ( $Y$ ) into individual desirability values ( $d$ ) with a one-sided limit  $Y \leq Y_{\max}$  or  $Y \geq Y_{\min}$  was performed using the following equation:

$$d = \exp[-\exp(Y')] \quad (1)$$

where  $Y' = b_0 + b_1$ . The coefficients  $b_0$  and  $b_1$  were calculated by assigning the corresponding desirability values  $d$  for two of the property values, preferably selected within the range  $0.2 < d < 0.8$ . The desirability curve (Fig. 1) were plotted in the ( $Y'$ ,  $d$ ) coordinates based on the equation of the desirability function. At the same time,  $Y_{\max}$  or  $Y_{\min}$  on the dimensional scales corresponded to 0 (zero) on the dimensionless scale  $Y'$ . The desirability scale (Fig. 1) was used to convert the response values ( $Y_1, Y_2, Y_3, Y_4$ ) into the dimensionless desirability function ( $d_1, d_2, d_3, d_4$ ), i.e. to find individual desirability values for the measured values of the optimization parameters  $Y_i$ .

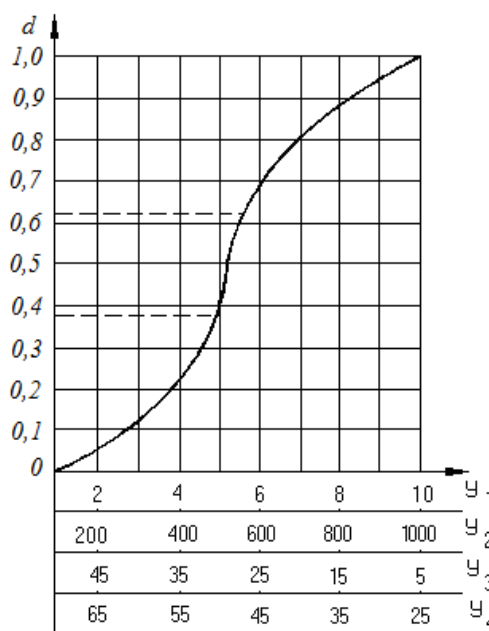


Fig. 1: The Desirability Function Scale of the Optimization Parameters for the Capsules "Nigelit"

Then the overall (generalized) desirability function values were calculated using the formula (2) as the geometric mean of individual desirability values found. It should be noted, this is a more successful approach towards optimization of the parameters for finished products (in our case capsules):

$$D = \sqrt[4]{d_1 d_2 d_3 d_4} \quad (2)$$

where  $D$  is overall (generalized) desirability function value;  $d_1, d_2, d_3, d_4$  - individual desirability function values.

The values of the overall (generalized) desirability function ( $D$ ) for the capsules "Nigelit" are presented in table 3.

Based on the generalized evaluation of the optimization parameters - the disintegration of capsules and the technological properties of the granular materials (model mixtures), carried out using a desirability function, the excipients can be arranged in the order of preference as follows:

- The type of fillers (Factor A) –  $a_3 > a_4 > a_1 > a_2$ ;
- The type of antiadherents (Factor B) –  $b_1 > b_2 > b_3 > b_4$ ;
- The type of disintegrants (Factor C) –  $c_2 > c_1 > c_3 > c_4$ ;

- The type of glidants (Factor D) –  $d_3 > d_1 > d_2 > d_4$ .

The optimal composition for capsules "Nigelit" was formulated based on the results of the mathematical method of experimental design and using a desirability function.

The most optimal combination of levels of factors - composition of the excipients that ensure the required indicators for the capsules "Nigelit" (table 3, composition No. 9) was selected based on the values of the overall (generalized) desirability function ( $D$ ) of the optimization parameters. The excipients included in this composition are listed in table 6.

**Table 6:** The Most Optimal Composition of the Excipients that Ensure the Required Indicators for the Capsules "Nigelit"

Name of the capsules	No. of the optimal composition	Excipients included in the optimal composition
"Nigelit"	composition No. 9 in table 3	MCC Arbocel A 300 (filler – $a_3$ ) Magnesium stearate(antiadherent– $b_1$ ) MCC Arbocel A 300 (disintegrant– $c_2$ ) talc(glidant – $d_3$ )

Based on the results of the mathematical method of experimental design, we recommend the following formulation and technology:

**Formulation:**

Nigella sativa seeds ground	-350mg
Zinc sulphate	-20 (equal to 7.3 mg of zinc)
MCC Arbocel A 300	-22mg
Talc	-4mg
Magnesium stearate	-4 mg
Average net weight of capsule	-00 mg

**Technological process:** A moisturizing liquid (water) is sprayed into the dry mixture during the mixing process of seeds ground of Nigella sativa and zinc sulphate with

"granule-forming" excipient- MCC Arbocel A 300(fillers)in order to form agglomerates - granules. The "drying" of granules is accomplished by adding a "drying" excipient –the rest amount of MCC Arbocel A 300 (disintegrant) into the mixer, during the continuous mixing process. Since the final moisture content of the product obtained by this granulation method usually does not exceed the final moisture content of the granules obtained by traditional wet granulation, we did not perform additional thermal drying of the granules. In the final stage, talc (glidant) and magnesium stearate (antiadherent) are added to the granules. The resulted compact granules have good technological properties which are presented in table 7.

**Table 7:** Results of the study of the technological properties of the granules for the capsules "NIGELIT"

No.	Studied indicators	Unit of measurement	Obtained results
1	Appearance		Dark brown granules with a strong, agreeable aromatic odor and a spicy, pungent taste
2	Particle-size distribution: +2500 -2500+1000 -1000+ 500 - 500+ 250 - 250	$\mu\text{m}$ , %	1.5 23.0 47.5 20.8 7.2
3	Bulk density untapped	$\text{kg}/\text{m}^3$	390
4	Bulk density tapped	$\text{kg}/\text{m}^3$	455
5	Flowability	$10^{-3} \text{ kg/s}$	5.5
6	Angle of repose	$^\circ$	38
7	Residual moisture	%, 70 $^\circ\text{C}$	4.2

As evidenced by the data in Table 7, in contrast to the mixture of active components, the granular material prepared according to the selected composition and technology has satisfactory technological properties.

Taking into account the amount of granular material to be encapsulated, its density, empty capsule volume capacity and the requirements for uniformity of the capsule contents the capsule size 00E was chosen to encapsulate the calculated dosage. [17, 20]. The process of filling the capsules with the granular material was performed using the capsule-filling machine MF 30.

#### IV. CONCLUSION

Thus, based on the results of study of technological properties of the the mixture of active components and the granular material prepared according to the selected composition and technology and using the mathematical method of the experimental design, the  $4 \times 4$  Latin square, an optimal composition was formulated and the rational encapsulation technology for the capsules "Nigelit" with an average net weight of capsule (weight of core material) 400 mg was developed.

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## Quality Assessment of Active Substances that Demonstrate Sedative Effect of “Flegmen” Syrup

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**Abstract-** As a result of the research, the amino acid and elemental composition of “Flegmen” syrup was determined, and it was found that the syrup contains 18 amino acids and 57 macro and micro elements. The accumulation in “Flegmen” syrup of a significant number of amino acids and useful minerals, along with active biologically active groups (flavonoids, saponins), allows us to objectively consider it as a valuable source of an effective sedative.

**Keywords:** *flegmen; amino acids; regel’s gooseberry; turkestan motherwort; peppermint; high performance liquid chromatography.*

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*Strictly as per the compliance and regulations of:*



# Quality Assessment of Active Substances that Demonstrate Sedative Effect of “Flegmen” Syrup

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**Abstract-** As a result of the research, the amino acid and elemental composition of “Flegmen” syrup was determined, and it was found that the syrup contains 18 amino acids and 57 macro and micro elements. The accumulation in “Flegmen” syrup of a significant number of amino acids and useful minerals, along with active biologically active groups (flavonoids, saponins), allows us to objectively consider it as a valuable source of an effective sedative.

**Keywords:** *flegmen; amino acids; regel's gooseberry; turkestan motherwort; peppermint; high performance liquid chromatography.*

## I. INTRODUCTION

Amino acids are organic compounds which are considered material unity of all tissues of human body. They are responsible for metabolic processes and energy metabolism, ensuring the functioning of the body. Amino acids directly affect the state of the nervous system, regulating mental activity, mood and sleep [2,5]. A deficiency of even one building block can worsen a person's health and lead to serious biochemical and physiological disorders in the body. Therefore, amino acid supplements are best taken in all cases when you load yourself - physically or mentally, but cannot fill the increased need in a timely manner solely from food. There are three groups of amino acids that are replaceable, irreplaceable and conditionally essential [11, 12].

The deficiency of essential amino acids is compensated by the human body with plant foods, and in the case of medical indications, in the form of medicinal preparations containing these compounds. Many amino acids are not only of great physiological importance, but are highly effective pharmacological substances. Replaceable can be independently synthesized in the body. Essential ones are not synthesized on their own and enter the body with food. Conditionally essential amino acids can be synthesized independently in the presence of essential amino acids. There are several optimal periods for taking amino acids. During training, amino acids will help improve

athletic performance, muscle growth and strength, as well as speed up recovery processes after exercise. During diets and drying, amino acids will help maintain muscles and health during forced starvation, as well as speed up the process of burning fat. IN periods of mental load of amino acids will increase intellectual productivity and relieve excessive psycho-emotional stress [9,10].

According to WHO, a significant part of the world's population suffers neuropsychiatric disorders during their lives. For the treatment of these types of disorders, sedative herbal preparations are often used, which is due to their wide range of action due to the presence of a complex of active substances in them, ease of use, ease of dosage, minimum contraindications and side effects.

With this in mind, from the plants most commonly used in the formulation of sedatives and having industrial stocks in the republic, namely, Regel's gooseberry, Turkestan motherwort, peppermint and licorice, we previously developed a collection and based on it a sedative syrup was obtained “Flegmen”[3,13].

Amino acids are an integral part of proteins, they perform one of the most important roles in the body. Almost all tissues are formed from them: skin, hair, ligaments, tendons. There are three types: replaceable, conditionally replaceable and irreplaceable. Non-essential amino acids are supplied to the body with food and can be synthesized in it. Essential amino acids must be supplied to the body from outside. Conditionally essential amino acids can be synthesized by the body from essential amino acids if necessary. There are twenty compounds in nature that form proteins. Non-essential amino acids include: glutamic acid, glycine, aspartic acid, serine, cysteine, tyrosine, alanine, proline. Essential amino acids are those amino acids that our body cannot produce on its own, they must be supplied with protein foods. Essential amino acids include: valine, isoleucine, leucine, threonine, methionine, lysine, phenylalanine, tryptophan, histidine. Conditionally essential amino acids include: arginine, tyrosine, cysteine. Each of them is responsible for a specific function [1,9,10].

As for the sources of obtaining biologically active substances, including amino acids, one of them is vegetable raw materials. In turn, it should be noted

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that biologically active substances of plant origin differ from synthetic compounds in that they are in plants in complexes easily absorbed by the body and biologically available concentrations [11,14]. Based on the above, this work, we decided to devote the study of one of the groups of such biologically active substances of the "Flegmen" syrup - amino acids in order to identify their possible participation in the manifestation of the sedative effect of the drug.

*The purpose* of the study was the study of the amino acid and mineral composition of the sedative syrup "Flegmen".

## II. EXPERIMENTAL PART AND METHODS

To this end, we decided to review the methods for the analysis of amino acids in various objects (primarily in drugs) described in the literature. At the same time, it was established that there are currently many methods for determining amino acids in various objects. Among them, the most common are methods for the determination of amino acids by reverse-phase and cation-exchange High Performance Liquid Chromatography (HPLC), as well as electrophoretic methods. Considering the high prevalence and a number of advantages over other methods, we preferred HPLC in the choice of the amino acid analysis method. In particular, the improvement of HPLC technology and its wide practical application makes it possible to solve the problems of separation and quantitative determination of very small amounts (10 mg/kg and below) of analyzed components in complex objects. However, the absence of chromophore groups in most amino acid molecules requires a derivatization step when using this method. At the same time, various reagents have been proposed for pre- and post-column derivatization.

Thus, in the works of a number of researchers on a C18 column in the gradient elution mode with a methanol-phosphate buffer mixture with fluorimetric detection, glutathione, glutamylcysteine, and 16 amino acids were simultaneously determined using o-phthalaldehyde as a derivatizing agent. And in the works of a number of authors, naphthalenedialdehyde was used in the determination of desmosine, isodesmosine, and 17 other amino acids. 11-dansyl derivatives of amino acid isomers were separated (UV detection) on a  $\beta$ -cyclodextrin column using a mobile phase methanol-phosphate buffer (pH=6.5) [9, 10].

As you know, for each new drug, a specific analysis method should be developed for it. When developing an HPLC technique, in order to obtain reliable results, it is most important to find the optimal conditions for the analysis, the main of which is the choice of the mobile phase (the phase with the highest selectivity with respect to active substances), the column size, the type and size of the sorbent particles, and the elution mode

(gradient or isocratic mode), detection method (conditions), standard, etc. All this, of course, requires appropriate research using various reagents and solvents, as well as a waste of time. However, when reviewing the literature, we came across the HPLC technique [6] used to determine similar substances in a similar object. This circumstance led us to the idea of testing this technique for our case, in order to establish its suitability for determining amino acids in "Flegmen" syrup and, if necessary, modifying it.

The object of the study was the "Flegmen" sedative syrup obtained on the basis of a plant liquid extract. The syrup obtained in this way is a thick solution of a light brown color, with a characteristic odor and a sweet, slightly icy taste [3,13].

The suitability of the chromatographic system was checked by the efficiency of the chromatographic column, the degree of separation of the peaks and the relative standard deviation.

Chromatography of the amino acids of "Flegmen" syrup and a standard mixture of amino acids with a known concentration was carried out sequentially under similar conditions. The results of the chromatographic analysis of the amino acids of the "Flegmen" syrup are shown in fig. one.

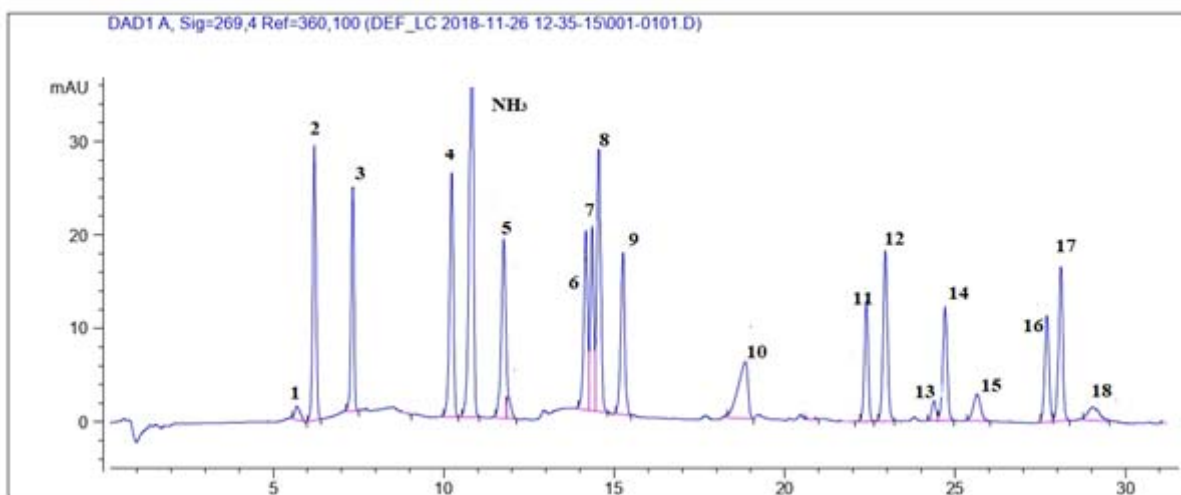


Fig. 1: Chromatogram of free amino acids of "Flegmen" syrup

1 - aspartic acid, 2 - glutamic acid, 3 - asparagine, 4 - serine, 5 - glutamine, 6 - histidine, 7 - threonine, 8 - glycine, 9 - arginine, 10 - alanine, 11 - tryptophan, 12 - methionine, 13 - tyrosine, 14 - valine, 15 - phenylalanine, 16 - isoleucine, 17 - leucine, 18 - lysine.

Identification and quantification of the amino acids contained in the study preparation was carried out by comparing the retention times and peak areas on the standard amino acid chromatogram with those on the amino acid chromatogram of the study preparation. Analysis of the obtained amino acid chromatograms of the test sample and the standard mixture showed the presence of several amino acids in the "Flegmen" syrup.

The results of the analysis with names, chemical formula and amino acid content are presented in Table 1.

Analysis of the obtained amino acid chromatograms of the test sample and the standard mixture showed the presence of several amino acids in the "Flegmen" syrup. The results of the analysis with names, chemical formula and amino acid content are presented in Table 1.

Table 1

No.	Amino acid name	Chemical formula	Content $\mu\text{g/ml}$
one	Aspartic acid	$\text{C}_4\text{H}_7\text{O}_4\text{N}$	0.0240
2	Glutamine acid	$\text{C}_5\text{H}_9\text{O}_2\text{N}$	0.0840
3	Asparagine	$\text{C}_4\text{H}_8\text{N}_2\text{O}_3$	0.0044
five	Glutamine	$\text{C}_3\text{H}_7\text{O}_3\text{N}$	0.0124
6	Histidine	$\text{C}_6\text{H}_9\text{O}_2\text{N}_2$	0.0038
7	Threonine *	$\text{C}_6\text{H}_9\text{O}_2\text{N}_3$	0.0292
8	Glycine	$\text{C}_2\text{H}_5\text{NO}_2$	0.0543
nine	argenin	$\text{C}_2\text{H}_5\text{O}_2\text{N}$	0.0668
10	Alanine	$\text{C}_6\text{H}_{14}\text{O}_2\text{N}_4$	0.0418
eleven	tryptophan	$\text{C}_{11}\text{H}_{12}\text{N}_2\text{O}_2$	0.0218
12	Methionine *	$\text{C}_5\text{H}_{11}\text{NO}_2\text{S}$	0.0421
13	Tyrosine *	$\text{C}_9\text{H}_{11}\text{NO}_3$	0.0112
fourteen	Valine *	$\text{C}_2\text{H}_5\text{O}_2\text{N}$	0.0177
15	Phenylalanine *	$\text{C}_6\text{H}_9\text{O}_2\text{N}_3$	0.0177
16	Isoleucine *	$\text{C}_6\text{H}_{13}\text{O}_2\text{N}$	0.0092
17	Leucine *	$\text{C}_6\text{H}_{13}\text{NO}_2$	0.0092
eighteen	Lysine HCl *	$\text{C}_6\text{H}_{14}\text{N}_2\text{O}_2$	0.0713
			<b>0.5209</b>

\*Essential amino acids

### III. RESEARCH RESULTS

According to the research data, "Flegmen" syrup contains 18 amino acids, which indicates a high biological value of the sum of amino acids of the studied liquid extract. Among the amino acids of the collection, glutamic acid predominates, which is used in the treatment of diseases of the central nervous system.

From this it follows that the amino acids of the studied syrup can participate in the manifestation of the pharmacological activity of the syrup.

Further research was aimed at studying the mineral composition of "Flegmen" syrup.

Trace elements have been known for a long time, but only very recently they have been recognized

as substances necessary for Life. Trace elements are "food mainly for the endocrine glands", more precisely, for enzyme enzymes, since they are catalysts for vital processes. In the impact on the body, all microelements are interconnected and interdependent. Human needs for these "metals of life" are very individual. Minerals make up only 4% of the mass of the human body. Half of this amount is part of the solid parts of the body: bones, teeth, nails, hair, soft tissues. The rest is in the blood, in the intercellular and intracellular fluid.

70-80% of our body mass is water and gases soluble in it - carbon, hydrogen, nitrogen and oxygen, and most of all in our body oxygen is about 60% of body weight, carbon - about 17%, hydrogen - about 10%, nitrogen - only 3% [3].

Micro and macro elements control metabolic processes, maintain the physical and chemical integrity of cells and tissues by maintaining characteristic bioelectric potentials. It is microelements that play the main role in the activity of enzymatic processes necessary for life. That is why their deficiency, as well as excess, will immediately affect human health. It should be noted that the intercellular space contains mainly sodium and calcium, and inside the cells - potassium and magnesium. If the balance between them is disturbed, a person develops various diseases, accompanied by swelling. In this case, the balance should be maintained both between sodium and calcium, and between potassium and magnesium [2,12].

It should also be noted that the minerals contained in plants are divided into two groups: the first, called macroelements, includes potassium, sodium, calcium, magnesium, manganese, silicon, chlorine, phosphorus; plant ash contains at least hundredths of a percent of these elements; the second, called trace elements, include: iron, copper, zinc, iodine, barium, etc. Their content in the ash is thousandths of a percent. Accumulation of trace elements in plants is often selective: different types of plants grow in the same soil conditions, and only some of them are able to concentrate certain microelements [4,7,14].

The determination of the elemental composition was carried out using a highly sensitive multi-element analysis method - mass spectral with inductively coupled plasma (ICP - MS) [15]. As a result of the analysis, the presence of 57 mineral elements was established in the syrup. According to the results obtained, 2 elements (Br and K) were contained in concentrations from 100 to 1000 mg/kg, 2 elements (Na and Mg) ranged from 10 to 100 mg/kg, 2 elements (P and Fe) ranged from 1 to 10 mg/kg, and below 1 mg/kg 51 elements (Fe, Zn, B, Cr, Al, I, Sr, Mn, Cu, Ca, Sc, Ba, Mo, Ni, Li, V, Se, Sb, Zr, Sn, Nb, As, Co, Ga, Ag, Cd, Ta, Cs, Te, W, Tl, Bi, Re, Nd, Ce, Pb, Hf, Y, Gd, In, Sm, La, Er, Eu, Dy, Pr, Lu, Ho, Tm, Au, Hg, Pt) [102; pp.206-213]. results and study of the elemental composition of "Flegmen" syrup are presented in table 2.

Table 2: Elemental composition of "Flegmen" syrup

Element	Soda in $\mu\text{g/ml}$	Element	Sod-e in $\text{mcg/ml}$	Element	Soda - e in $\text{mcg/ml}$
Br	4015	Li	0.016	Nd	0.00018
K	2755	V	0.01	Ce	0.00018
Na	51.9	Se	0.011	Pb	0.00015
mg	16.5	Sb	0.005	hf	0.00013
P	1.96	Zr	0.003	Y	0.0001
Fe	1.41	sn	0.002	Gd	0.00007
Zn	0.22	Nb	0.0016	In	0.00007
B	0.16	As	0.0012	sm	0.00006
Cr	0.14	co	0.0011	La	0.00006
Al	0.12	Ga	0.001	Er	0.00006
I	0.125	Ag	0.0008	Eu	0.00005
Sr	0.11	CD	0.0007	Dy	0.0005
Mn	0.08	Ta	0.0007	Pr	0.00004
Cu	0.08	Cs	0.00055	Lu	0.00003
Ca	0.07	Te	0.0004	Ho	0.000019
sc	0.03	W	0.00046	Tm	0.00001
Ba	0.042	Tl	0.00035	Au	0.0000039
Mo	0.033	Bi	0.00033	hg	0.0000023
Ni	0.01	Re	0.00026	Pt	0.0000023

As the data in table 2 show, "Flegmen" syrup contains 57 elements. Of these elements, calcium, magnesium, potassium, sodium and chlorine, which are part of the cell in the form of ions, are vital. The listed elements are included in the group of macronutrients.

Macronutrients in the syrup, the largest quantities are: bromine, potassium, sodium, magnesium, phosphorus, iron, zinc, boron, chromium. Elements found in syrughaving a positive effect on the vital activity of the organism, to a certain extent, contribute to an increase



in the pharmacological value of this medicinal vegetable syrup due to the combination with its main biologically active substances [10].

The detected elements according to the degree of decrease in their content can be represented as the following series: Br> K> Na > Mg >P >Fe >Zn >B >Cr > Al> I> Sr > Mn= Cu> Ca >Sc >Ba>Mo>>Ni >Li >V >Se >Sb >Zr >Sn > Nb > As > Co > Ga >Ag>Cd= Ta> Cs> Te > W > TI > Bi, >Re >Nd= Ce > Pb > Hf > Y > Gd=In >Sm> La > Er >Eu > Dy > Pr >Lu > Ho >Tm > Au > Hg= Pt

When determining the elemental composition of the syrup, special attention is paid to the content of toxic heavy metals - lead, cadmium and mercury, which the FAO and WHO Joint Commission on the Food Code (Codex Alimentaris) refers to the number of components subject to priority control in international food trade [8,9]. It is shown that the content of toxic heavy metals in the syrup is within the limits allowed by SanPin 0193-06[4]. Comparison of the concentrations of these metals in the studied preparation with their clarks showed that their content practically corresponds to uncontaminated territories, which indicates the environmental safety of raw materials.

Thus, for the first time by the ICP - MS method, the mineral composition of "Flegmen" syrup was determined, in which the content of 57 elements was found. Elements such as bromine, potassium, sodium, magnesium, phosphorus, zinc have been found, which have a pronounced sedative effect and have a beneficial effect on nervous tissue, restoring performance after emotional and physical stress. The data obtained allow us to conclude that the elemental composition of the syrup is very diverse and, accordingly, can have a complex effect. It has been established that the content of toxic heavy metals lead, cadmium and mercury does not exceed the permissible values, which indicates the environmental friendliness and safe use of the syrup in medical practice.

Also, from table 2 it can be seen that in the "Flegmen" syrup such elements were found that are involved in sedative activity.

Magnesium deficiency, even if not too great, can be the cause of heart disease. A serious lack of this mineral leads to disastrous consequences - as a rule, to heart attacks. Lack of magnesium leads to anxiety, fear, confusion, depression. Also, there is hyperactivity, nervousness, stepping from foot to foot, jumping gait, sharpness of movements. Loss of balance, dizziness, fainting, weakness in the arms and legs, blood pressure disorders, cold extremities. The trace element magnesium promotes the absorption of calcium. Bromine is involved in the regulation of the activity of the thyroid gland, as it is a competitive inhibitor of iodine.

The lack of bromine in food leads to insomnia, growth retardation and a decrease in the number of erythrocytes in the blood [2,11]. Phosphorus and

bromine have a pronounced sedative effect and have a beneficial effect on the nervous tissue, restoring performance after emotional and physical stress [2,11,12].

Lack of iodine contributes to the development of Graves' disease (goiter). Children and adolescents require more iodine than adults. Iodine is used in atherosclerosis, treatment of syphilis in the tertiary period, inflammatory processes of the respiratory tract, chronic mercury and lead poisoning, to prevent and treat goiter [2,11]. Potassium iodide is prescribed for mastopathy of the mammary gland and other neoplasms in the endocrine glands. Iodine has a sedative (calming) effect on a person, increases mental abilities. Iodine is necessary for the synthesis of the thyroid hormone - thyroxine, as well as for the creation of phagocytes - patrol cells in the blood, which must destroy debris and foreign bodies. Phagocytes capture and digest microorganisms, defective cells.

Lithium prevents the development of neuropsychiatric diseases and has a positive effect on the treatment of schizophrenia [2,11,12]. Zinc deficiency is of exceptional importance, as it not only leads to underdevelopment of the nervous and reproductive systems, but is also deeply linked to immunodeficiency problems. T-lymphocytes in conditions of zinc deficiency are inactive.

Potassium is a very common mineral found in many foods. The best sources of potassium are plant products, especially dried fruits and berries, nuts, seeds, Jerusalem artichoke, potatoes, radishes, cabbage, green vegetables, oatmeal, beets, bananas, bread, currants, tomatoes. Symptoms of potassium deficiency are muscle weakness, heart problems and mental disorders. Low potassium intake can lead to sodium retention and high blood pressure. A potassium-rich diet has been linked to beneficial effects on cardiovascular health. The macroelement potassium is needed, first of all, for the transmission of nerve impulses, to maintain the acid-base balance of the blood, for normal carbohydrate metabolism, to ensure muscle contraction. Its need increases primarily with vomiting and prolonged diarrhea, with profuse sweating, with diuretics, with increased excretion of potassium in the urine, which can be caused, as well as excessive amounts of sodium, coffee, sugar and/or alcohol consumed, or low blood sugar levels. blood.

The macronutrient sodium is primarily needed for normal water exchange between blood cells and tissues, to maintain the acid-base balance in the body, to transmit nerve impulses, to ensure muscle contraction.

A profound lack of sodium can lead to coma and death. Excessive consumption of this macronutrient burdens the kidneys, causes edema (normal water exchange between blood cells and tissues is disturbed), can cause an increase in blood pressure, and leads to

excessive excretion of water and potassium in the urine (which, however, does not relieve edema). Dietary sodium deficiency usually does not occur. Acute deficiency can occur with profuse sweating in combination with the consumption of large amounts of non-sodium fluids, or as a result of vomiting and diarrhea. Symptoms are muscle cramps, lack of appetite, malabsorption of nutrients.

#### IV. CONCLUSION

Thus, as a result of the research, the amino acid and elemental composition of the "Flegmen" syrup was determined, while it was found that the syrup contains 18 amino acids and 57 macro and micro elements. Among the amino acids of the collection, glutamic acid predominates, which is used in the treatment of diseases of the central nervous system. Among the macronutrients, such elements as lithium, phosphorus, iodine, magnesium, bromine were found, which have a pronounced sedative effect and have a beneficial effect on the nervous tissue, restoring performance after emotional and physical stress. The data obtained allow us to conclude that the amino acid and elemental composition of the syrup is very diverse and, accordingly, can have a complex effect.

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## Review on Emulsomes as Carriers for Drug Delivery

By Bandaru Hemanth Kumar, Shaik Farooq Ahmed & Prasanthi D

**Abstract-** This review determines the introduction to emulsomes, need to the invention, advantages, disadvantages, formulation of the emulsomes, methods of preparation and application of emulsomes. In the recent years attention has been focused on development of vesicular drug delivery system. These emulsomes provide the drug release in a controlled and sustained manner up to 24 hours, whereas the liposomes have shown release up to the mark of 6 hours. Emulsomes comes under the category of the vesicular drug delivery system and these are mainly developed for the purpose to overcome poor bioavailability, protection from harsh gastric environment and from gastric enzymes, which mainly degrade the drug molecules. The success of the emulsomes is for the delivery of drugs to fight against viral infections, fungal infections, dermal therapy, cancer, auto immunity. Mainly the drug is enclosed in the emulsomes and provide existence of drug in systemic circulation. Emulsomal based formulations of genetic drugs, antisense oligonucleotides and plasmids for gene therapy having proper and clear potential for systemic utility are increasingly available.

**Keywords:** emulsomes, liposomes, emulsions, preparation methods of emulsomes, applications of emulsomes.

**GJMR-B Classification:** DDC Code: 610.3 LCC Code: R121



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# Review on Emulsomes as Carriers for Drug Delivery

Bandaru Hemanth Kumar <sup>α</sup>, Shaik Farooq Ahmed <sup>σ</sup> & Prasanthi D <sup>ρ</sup>

**Abstract-** This review determines the introduction to emulsomes, need to the invention, advantages, disadvantages, formulation of the emulsomes, methods of preparation and application of emulsomes. In the recent years attention has been focused on development of vesicular drug delivery system. These emulsomes provide the drug release in a controlled and sustained manner up to 24 hours, whereas the liposomes have shown release up to the mark of 6 hours. Emulsomes comes under the category of the vesicular drug delivery system and these are mainly developed for the purpose to overcome poor bioavailability, protection from harsh gastric environment and from gastric enzymes, which mainly degrade the drug molecules. The success of the emulsomes is for the delivery of drugs to fight against viral infections, fungal infections, dermal therapy, cancer, auto immunity. Mainly the drug is enclosed in the emulsomes and provide existence of drug in systemic circulation. Emulsomal based formulations of genetic drugs, antisense oligonucleotides and plasmids for gene therapy having proper and clear potential for systemic utility are increasingly available. The worldwide market for drug delivery systems is growing at an ever-increasing rate and is being fueled by several significant needs, the commercial necessity of extending product life and product portfolios, the clinical need to enhance drug safety and patient compliance and the technological challenge of delivering new therapeutics. Exploitation of new advances in drug delivery technology will give pharmaceutical companies a significant competitive advantage in an increasingly demanding marketplace.

**Keywords:** emulsomes, liposomes, emulsions, preparation methods of emulsomes, applications of emulsomes.

## 1. INTRODUCTION

### a) Emulsomes

Emulsomes are nanosize in range compared with the other vesicular drug delivery system such as niosomes, pharmacosomes, ethosomes. Due to their reduced size, they can be used to increase the bioavailability of drug and as the best carrier for the intravenous delivery as well as the oral drug delivery[1]. Oral route is the best route to reduce the number of adverse effects. Emulsomes are novel oral drug delivery systems which carries lipophilic drugs within it. Emulsomes are lipoidal vesicles which contains solid fat

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core surrounded by phospholipid bilayer. They are the liposomes with extra single inner phospholipid layer which contains solid fat. The drug release pattern by emulsomes is sustained and slow release and it is also soluble in aqueous phases and can be easily circulated through blood[2]. The Emulsome nanocarrier technology is a lipid-based drug delivery system designed to act as a vehicle for drugs with poor water solubility. Emulsome particles consist of a microscopic lipid assembly with an internal fat core, which dissolve the water-insoluble drugs in the absence of any surfactant or solvent.

### b) Need for the invention

Undesirable side-effects are often produced when water-insoluble vehicles are used for the parenteral administration for example thrombophlebitis, hemolysis, or blood coagulation. The potential carriers for fat soluble materials are liposomes and o/w emulsions which minimize such undesirable side effects. However, there are many problems with stability and drug loading capacity which have been reported using either of these delivery systems[3].

### c) Liposomes

It consists of one or more concentric phospholipid bilayers, separated by water or aqueous compartments, range from 20nm to 10  $\mu$ m.

They are

1. SUV (20-100nm)
2. LUV (> 100nm)
3. REV (0.5 $\mu$ m)
4. MLV (2-10 $\mu$ m)

Effective for localized sustained release of drugs in tissues [4].

### Drawbacks of liposomes

1. Unilamellar Vesicles- low content of lipid molecules so
  - Low drug loading capacity for lipophilic compounds
  - More suitable for entrapment of water –soluble materials
2. Amount of drug that can be contained therein is limited.
3. MLV liposomes- not appropriate for I.V due to large size.
4. Difficulties in preparation of acceptable liposomal formulations with long-term stability and high drug loading [2].

d) *Emulsions*

Emulsions are defined as “heterogeneous systems of one liquid dispersed in another liquid in the form of droplets usually exceeding 1  $\mu\text{m}$  in diameter”. The two liquids are immiscible and are chemically not reactive or slowly reactive. An emulsion is a thermodynamically unstable dispersed system. This instability causes reduction in its free energy by separating the dispersed droplets into two liquid phases.

Evidenced of emulsion instability during storage:

- Creaming
- Flocculation
- Coalescence [5]

*Drawbacks*

1. Micro-droplets of size less than 1 $\mu\text{m}$  should be achieved to prevent the formation of emboli in blood vessels.
2. Emulsifiers must be coated to lower the free energy at the interface and decrease the tendency of droplets to coalesce. These emulsifiers produce harmful side effects upon injection into the body.
3. It has detergent characteristics because most of them are hemolytic agents which act as membrane solubilizers.
4. Limited formulation options due to restricted emulsifiers for safe parenteral injection.
5. The water insoluble drugs such as phenytoin, amphotericin B, cyclosporin, miconazole, diazepam, etoposide, etc makes the formulation difficult for intravenous use.
6. These drugs are marketed in co-solvent systems such as polyethylene glycol or propylene glycol-ethanol-benzyl alcohol mixtures, which have shown toxicity problems, such as thrombophlebitis on injection.
7. Alternatives to cosolvent systems are micellar solutions or emulsions, but the presence of toxic surfactants in these systems makes them undesirable for intravenous administration[5].

*Summary of the invention*

To provide pharmaceutical compositions comprising nano-emulsions of particles comprising of lipid core composed of lipid which is in a solid or liquid crystalline phase atleast 25° C, stabilized by at least one phospholipid envelope, for parenteral, oral, rectal, intranasal or topical delivery of both fat-soluble and water-soluble drugs.

This is solid fat nanoemulsion or “EMULSOMES”.

Emulsomes, having the characteristics of both liposomes and emulsions [1]

## II. FORMULATION

## 1. Composition of lipid core

It exhibits solid (or) liquid crystal or mixed solid and liquid crystal phases at room temperature (25°C) when measured in bulk. Lipid compositions suitable for use as the core component of emulsomes may be characterized as being in the solid or liquid crystalline phase at least about 25°C., when measured in bulk form without incorporation into emulsomes. Some lipid compounds present in a mixture optionally may be fluids at 25°C. When pure provided that the lipid mixture as a whole is solid or liquid crystalline in bulk at 25°C. In preferred compositions, at least 90% of the individual lipid compounds present in the core are solids or liquid crystals at 25°C when measured in pure bulk form.

Phase determination is performed on the bulk lipid, incorporation into the emulsome core. The macroscopic phase determination on a bulk sample may be made on a melting apparatus or by spectroscopic means, such as IR, NMR, or fluorescence intensity or anisotropy. Bulk phase determination of an existing emulsome preparation may be performed by first extracting the core lipids, then measuring.

It consists of - triglycerides,

- Monoesters,
- Cholesterylesters & cholesterol,
- Antioxidants,
- Protein components [6]

a) *Triglycerides*

Available as synthetic triglycerides or mixture of several triglycerides. Fats isolated from natural sources usually are available only as mixtures of triglycerides. Such natural mixtures are suitable for preparation of emulsomes, provided that the melting characteristics of the mixture are such that they exhibit a solid or liquid crystal phase at 25°C.

Triglycerides, which are solid at 25°C have fully saturated fatty acid chains which are incapable of undergoing peroxidation reactions.

Examples of solid fats suitable for the preparation of emulsomes are:

Triglycerides composed of natural, even-numbered and unbranched fatty acids with chain lengths in the C10-C18 range, or microcrystalline glycerol triesters of saturated, even-numbered and unbranched fatty acids of natural origin such as tricaprins, trilaurins, trimyrists, tripalmitins, and tristearins.

Partially hydrogenated vegetable oils may be used to prepare emulsomes which are free of cholesterol or cholesteryl esters [7].

In some the lipid of the hydrophobic core may have a solid to fluid phase transition (melting) temperature between 25°C and physiological temperature (37°C) when measured in bulk. For example, tricaprins melts at 35°C-37°C., and is wholly or



predominantly in the fluid phase at physiological temperature. Tricaprin may be used to form an excellent lipid core for nanoemulsions. Lipid core may be composed of lipid, which is in solid phase at 37 °C.

Ex: higher saturated triglycerides-tripalmitin and tristearin

#### b) Monoesters

The lipid core may contain monoesters of fatty acids such as waxes

Ex: Esters from beeswax and spermaceti-cetyl palmitate

Preferred waxes are made from saturated or monounsaturated fatty acids and saturated or unsaturated fatty alcohols.

Ex: Arachidyl oleate

Other monoesters include solid monoglycerides such as glyceryl monostearate, and fatty acid esters of short chain alcohols such as ethyl stearate [8].

#### c) Cholesteryl esters & cholesterol

These can be incorporated into the lipid core or the surrounding phospholipid envelope. cholesterol has a polar alcohol group, it tends to incorporate into the envelope monolayers or bilayers rather than into the lipid core itself, and should be considered a component of the phospholipid envelope rather than of the core [9].

Preferred cholesteryl esters are those of saturated or monounsaturated long chain fatty acids, such as palmitoyl or oleoyl, respectively.

Cholesteryl esters may be present in levels up to 50 mol % relative to the triglyceride or other solid lipid core component [10].

#### d) Antioxidants

Lipid core may contain one or more antioxidants.

The need for antioxidants may be lessened by preparing the lipid core from saturated fatty acids.

Ex: Alpha tocopherol & its derivative  
Butylated hydroxytoluene

#### e) Protein components

Lipid particles of the invention preferably do not contain serum apolipoproteins such as apo B, apo AI, apo AII, or apo E.

The apo B protein has the effect of targeting intravenously administered lipid particles to certain cellular receptors, such as the LDL receptor on hepatocytes and certain other cells.

Other proteins and peptides optionally may be present in emulsomes.

Examples of such peptides and proteins may be cyclosporin, luteinizing hormone releasing hormone (LHRH) and its analogs, calcitonin, insulin, and other synthetic or recombinant peptides.

An example of natural protein is collagen, which may be used to prepare emulsomes with controlled or sustained release properties.

#### 2. Phospholipids: Constitute the surrounding envelope of emulsomes

Ex: 1. Natural phospholipids - soybean lecithin, egg lecithin, phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, phosphatidic acid, diphosphatidylglycerol, Cardiolipin, phosphatidylserine, phosphatidylcholine, sphingomyelin.

2. Synthetic phospholipids- dimyristoyl & distearoyl phosphatidylglycerol, dimyristoyl & dipalmitoyl phosphatidylcholine, Hydrogenated lecithin & phospholipids.

The phospholipid component may be either saturated or unsaturated, and may have a gel to fluid phase transition temperature either above or below 25°C.

Ex: egg or soy PC – below room temp  
dimyristoyl PC – slightly below room temp  
distearoyl & dipalmitoyl PC –above room temperature  
emulsomes prepared with phospholipids which are in the gel phase at 37°C are expected to have more rigid bilayer envelopes and longer circulation time in plasma.

Emulsomes may be prepared with molar ratios of phospholipid to total lipid in the range of 0.1 to 0.75 (10 to 75 mol %), more usually 0.1 to 0.5 (10 to 50 mol %). The molar ratio of phospholipid to core lipid typically may be in the range of 0.1:1 to 2:1, usually 0.1:1 to 1:1, often 0.2:1 to 0.9:1, frequently 0.2:1 to 0.8:1, and commonly 0.25:1 to 0.6:1.

On a weight basis, the ratio of phospholipid to core lipid usually falls in the range 0.5:1 to 1.5:1, and frequently 0.6:1 to 1.2:1. [1]

3. Non-natural surfactants: optionally may be incorporated into emulsomes in small amount as less than 0.1% to less than 10% (mol/mol) of total surfactants.

The increasing concentrations of synthetic surfactants progressively decrease the particle size, and higher concentrations than those used are expected to result in formation of micelles (1-10 nm diameter).

4. Negatively charged lipids: These are added to the lipid phase of emulsomes to increase the zeta potential of the composition, thus stabilizing the particles.

Incorporation of these negatively charged lipid compounds in emulsomes results in the formation of phospholipid bilayers with opposing charges, thus increasing the loading of water-soluble molecules in the aqueous compartments formed by the phospholipid bilayers surrounding the lipid core.

Inclusion of negatively charged lipid molecules in emulsomes is to reduce the likelihood of particle aggregation, which minimizes destabilizing processes such as coalescence, flocculation, or fusion. Aggregation is prevented by the repulsive forces between the approaching particles.



Ex: negatively charged lipid molecule-oleic acid negatively charged phospholipid- phosphatidylglycerol, phosphatidic acid, phosphatidylserine, phosphate-dylinositol

Range is 0 to 30-mol % relative to total phospholipid & charged lipid.

#### 5. Incorporation of drugs

Water insoluble compounds – incorporated by dissolving drug in suitable organic solvent along with other ingredients.

Water-soluble drugs- by dissolving in aqueous medium.

#### *Categories of drugs incorporated*

Antifungal, antiepileptic & anticonvulsant drugs, beta-adrenergic blockers, aids drugs, anti-anxiety agents, anti-depressants, corticosteroids, anabolic steroids, estrogens & progesterones. [11]

### III. PHARMACEUTICAL PREPARATIONS OF EMULSOMES

#### a) Method

##### 1) Lipid film formation (Handshaking method)

Surfactants/lipids are casted as layers of film on their organic solution using flask rotary evaporator under reduce pressure (or) by hand shaking. The casted films are dispersed in aqueous. Hydration is done with constant hand shaking. The lipids will swell and get peeled off from the walls of round bottom flask at slightly above the phase transition temperature of surfactants used for specific period of time. Swelling of lipid and dispersion of casted lipid film is done by manual hand shaking or by exposing the film to a steam of water saturated nitrogen for 15 minutes, followed by swelling in the aqueous medium without shaking. Hand shaking method produce multi lamellar vesicles (MLV) and non-shaking method produced large unilamellar vesicles (LUVs).

##### 2) Reserve phase evaporation

This technique is comprised of two steps. First prepare a water-in-oil emulsion of phospholipids and buffer in excess organic phase. Second remove organic phase under reduced pressure. The two phases of phospholipids and water are usually emulsified by mechanical methods. Remove the organic solvent under vacuum, it causes the phospholipid coated water droplets to combine to form a gel-like matrix. Further continual removal of organic solvent under reduced pressure causes the gel like matrix to form into a paste of smooth consistency, which is a suspension of LUV. Drug entrapment efficiency is achieved up to 60-65%. This method is used to encapsulate both small and large molecules. Avoid the exposure of drug to be encapsulated to organic solvents and to mechanical agitation as less as possible. Phospholipids are dissolved in organic solvents such as chloroform, isopropylether, or mix two organic solvents to adjust the

density to unity that is closer to the density of aqueous phase. Biologically active molecules such as enzymes, protein pharmaceuticals and RNA type molecules may undergo conformational changes, protein denaturation, or breakage of DNA strands due to the harsh conditions of organic solvent exposure and mechanical agitation[7].

##### 3) Ethanol injection method

It is the alternative method used for the preparation of small unilamellar vesicles (SUVs). An ethanol solution of surfactant is injected rapidly through a fine needle into excess of saline or other aqueous medium. Vaporize the ethanol for the formation of vesicles. Narrow distribution of small liposomes (under 100 nm) can be obtained by simply injecting an ethanolic lipid solution in water, i.e. in one step, without extrusion or sonication. This method is a suitable technique to obtain the spontaneous formation of emulsomes with small average radius. Alternatively, the lipid or lipid mixture is dissolved in alcoholic solvent and an aliquot of 200, 500, or 600 ml fast injected at room temperature, 1 ml syringe into the dispersant solution, which contains water or saline solution, of 9.8 ml further diluted to 1:50, 9.5 ml diluted to 1:20 or 9.8 ml diluted to 1:17, respectively. The solution was then vigorously hand-shaken for 20-30 seconds. After that the ethanol solution is fast-injected in a 5% glucose solution. The vesicles had shown average diameter of about 60 nm and may be stable for at least one week[6].

##### 4) Cast film method

Mix the phospholipids and triglycerides in a weight ratio of 0.5:1.0 where triglycerides have a solid to liquid phase transition temperature of greater than 25°C. Suspend the mixture in an aqueous solution at a temperature below the solid to liquid transition temperature in order to reduce the suspension to yield emulsomes. These emulsomes comprise a nano-emulsion of liquid particles having a mean particle diameter between 10-250 nm usually within the range 20 to 180 nm usually and frequency within range 50-150 nm. The size range is determined on a weight percentage basis rather than a particle number basis. Usually, the lipid component may be volatile and chemically un-reactive volatile organic solvent such as dichloromethane or diethylether. Remove the solvent under reduced pressure in a rotary evaporator or under stream of inert gas. The resulting lipid film is then hydrated and dispersed by covering and shaking with an aqueous solution. If the drug component were not included in the organic solution, they may be added to aqueous hydration solution. Size the lipid suspension or dispersion at 800 pressure bars by high shear homogenizer.

##### 5) Detergent removal technique

Phospholipids and a detergent are mixed together to form micellar mixtures. The detergent is

removed from the preparation while the micelles progressively become richer in phospholipid content and the lipids come together to form single bilayer vesicles. Methods such as column chromatography, dialysis or adsorption onto bio beads used to remove the detergent from the preparation. The dialysis technique was first reported for reconstituting biological membranes solubilized with detergents. This method is also applicable for the preparation of emulsomes. Commonly used detergents here are those with high critical micelle concentration. Ex: sodium cholate, sodium deoxycholate, and octylglycoside. In this technique detergent is removed by a flow through dialysis cell from phospholipid detergent mixture. Reports were found that this technique yielded homogeneous population of single layered emulsomes with mean diameters of 50-100nm[4].

### APPLICATIONS OF EMULSOMES

1. Entrapment of water insoluble drugs
  - Neuroprotectant drug HU-211
2. Encapsulation of water –soluble drugs
  - Adaprolol- Maleate
3. For controlled release
4. As blood substitutes or oxygen carriers
  - Stable blood–substitute perfluorodecaline formulation
  - Perfluorotributylamine formulation
5. Can be lyophilized
6. In anti-viral therapy (anti HIV)
  - AZT- CDS in emulsomes
  - Brain enhanced delivery of AZT-Q by AZT-CDS-emulsomes
7. For ophthalmic use
  - 1% Indomethacin
8. For topical use as creams
  - 1% Indomethacin
  - Diclofenac & ketoprofen
9. In anti-fungal therapy
  - Miconazole
  - Amphotericin-B
10. Antiepileptic & anticonvulsant
  - Diazepam
  - Phenytoin
11. For sustained & targeted delivery
  - Zidovudine – to liver
12. For immunization
  - HIV-1 neutralising antibodies in genital & respiratory tracts of mice intranasally immunized with oligomeric gp160 formulated in emulsome[3]

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# Usage of Nivolumab- Platinum Containing STAT1 Molecule for Suppression PD-1/PD-L1 Genes in PD-1/ PD-L1 Expressing Cancer Cells

By Waleed O. Atta

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**Abstract-** Blockage of PD-1 proteins by immune checkpoint inhibitors showed an accepted therapeutic effect in cancer. But; tumor microenvironment exerts its antitumor influence by various mechanisms. Malignant cells have the ability of PD-1/PD-L1 protein over synthesis, which can be a defense action against immune checkpoint inhibitors and immunotherapy. Binding nivolumab with platinum-containing STAT1 will be used to reduce PD-1 genetic level. Nivolumab has the option of endocytosis, while STAT1 is the transcription factor that binds to the DNA, specifically PD-1 gene. STAT1 is the activated protein in response to multiple cytokines stimulation of cancer cells, which are the same for increasing PD-1/ PD-L1 upregulation. The used STAT1 in our therapeutic strategy is the activated form and loaded with platinum particles for damaging DNA bases in PD-1 promoter regions upon translocation to the nucleus. STAT1-platinum molecule is connected to nivolumab Fc region by solamargine polymer for selective cancer cell targeting.

**Keywords:** A5 complex, IFN- $\gamma$  endocytosis, cytokines, solamargine, phosphorylation.

**GJMR-B Classification:** DDC Code: 784.2 LCC Code: M3.1



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Waleed O. Atta

**Abstract-** Blockage of PD-1 proteins by immune checkpoint inhibitors showed an accepted therapeutic effect in cancer. But; tumor microenvironment exerts its antitumor influence by various mechanisms. Malignant cells have the ability of PD-1/PD-L1 protein over synthesis, which can be a defense action against immune checkpoint inhibitors and immunotherapy. Binding nivolumab with platinum-containing STAT1 will be used to reduce PD-1 genetic level. Nivolumab has the option of endocytosis, while STAT1 is the transcription factor that binds to the DNA, specifically PD-1 gene. STAT1 is the activated protein in response to multiple cytokines stimulation of cancer cells, which are the same for increasing PD-1/ PD-L1 upregulation. The used STAT1 in our therapeutic strategy is the activated form and loaded with platinum particles for damaging DNA bases in PD-1 promoter regions upon translocation to the nucleus. STAT1-platinum molecule is connected to nivolumab Fc region by solamargine polymer for selective cancer cell targeting.

**Keywords:** A5 complex, IFN- $\gamma$  endocytosis, cytokines, solamargine, phosphorylation.

## I. BACKGROUND

### a) PD-1/ PD-L1/PD-L2 Levels and Functions

PD-1 is a surface glycoprotein and is presented on CD4+ and CD8+ T cells, natural killer [NK] cells, natural killer T [NKT] cells, B cells, macrophages, and dendritic cells [DC] subsets. Also; PD-1 is expressed on tumor cells and TAMs [tumor associated macrophages] of many cancer types such as melanoma, non-small cell lung cancer, and head and neck squamous cell cancer [Baumeister et al. 2016]. PD-1 surface receptors should be suitable because it controls the immune balance towards self-antigens. Its deficiency or increased level causes altered immune response, lethal immune response and autoimmune disorder. PD-1 deficiency in murine models by genetic knockdown or blocking its signaling pathway results in serious immunopathology during acute infection via elevated levels of cytokines that result in tissue damage [Barber et al. 2006, Frebel et al. 2012]. Other harmful possibilities can occur, such as autoimmune dilated cardiomyopathy and autoimmune encephalomyelitis. [Sage et al. 2018] PD-1 has an inhibitory function on binding with PD-L1 and PD-L2 expressing cells

[Latchman et al. 2001]. PD-L1 and PD-L2 receptors are expressed on hematopoietic cells such as CD8+, CD4 + T cells, B cells, dendritic cells, macrophages and non-hematopoietic cells like hepatocytes, vascular endothelial cells, epithelial cells, myocytes, pancreatic islet cells, placenta and eye cells. Also, PD-L1 and PD-L2 are expressed on tumor cells and stromal tumor cells [Sun et al. 2018]. PD-1 inhibitory signals play a critical immune modulatory response by induction regulatory [Treg] and natural [T reg]. As result, immune modulatory molecules, such as anti-inflammatory cytokines transforming growth factor- $\beta$  [TGF- $\beta$ ] and interleukin-10 [IL-10], are secreted [Attanasio et al. 2016]. Activated Treg cells show high PD-1 levels, and their blockage will inhibit Treg cells' essential function.

PD-1 is overexpressed on M1 and M2 macrophages within the tumor tissue that represent tumor-associated macrophages [TAMs]. M1 macrophages have an early tumorigenic effect, while M2 macrophages stimulate metastasis [Tamura et al. 2018, Pollari et al. 2018] PD-1 receptors within the tumor different cells inactivate T cells, B cells, Natural killer cells and dendritic cells, by that way it inhibits the phagocytic action of T cells and other cellular immune response against tumor cells [Gordon et al. 2017]. The cytoplasmic tail of PD-1 entails two structural motifs: ITIM and ITSM. Once binding to PD-L1/PD-L2, the tyrosine residues are phosphorylated, which permits the efficacy of cytoplasmic tyrosine phosphatases such as SHP2. These phosphatases attenuate the signal of the TCR and CD28 [Berraondo, 2019]. PD-1 expressing tumor cells by that mechanism can convert CD8+cytotoxic cells into exhausted cells. PD-1 can stimulate and induce T regulatory cells to consider tumor antigens as self-antigens and escape from phagocytosis within the tumor microenvironment [Jiang et al. 2015]. Also, PD-1 disturbs T cell metabolism by glycolysis suppression and lipolysis stimulation [Patsoukis et al. 2015].

### b) PD-L1/ PD-1 Overexpression and Association with Stat1 Level

Tumor cells can induce stromal cells and TAMs to express PD-L1 directly by cell to cell contact or indirectly through secretion specific mediators such as IL-4, IL-6, IL-10, IL-13, CXCL8, SPP1 and IFN- $\gamma$  [Lu et al.

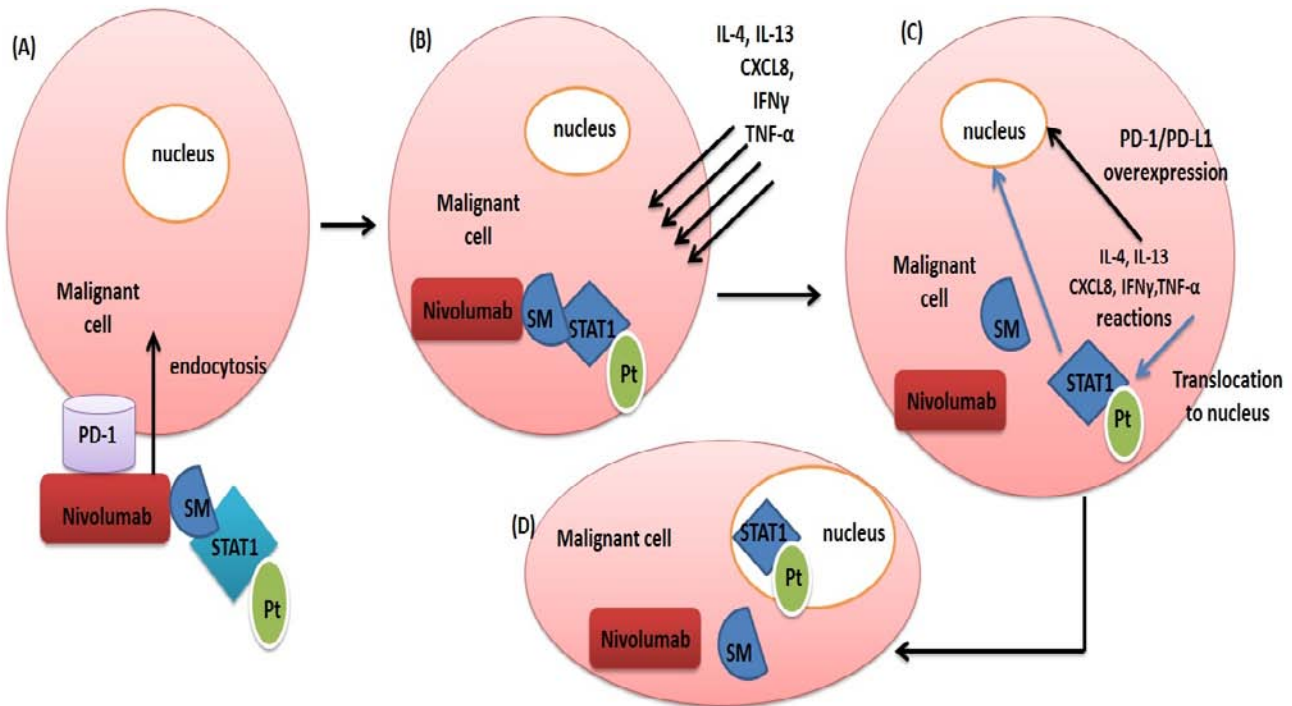
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2019]. In that way, tumor cells recruits surrounding cells for macrophage resistance by PD-L1 increased transcription. STAT1 is activated by the same activators of PD-L1 such as IL-4, IL-13 [Wang et al. 2004], CXCL8 [Chen et al. 2019]  $\alpha$  and TNF-[Wang et al. 2000].

The number of PD-1 membrane receptors is increased by IFN- $\gamma$  that activates Janus Kinases [JANs] that phosphorylate STAT1; in turn, activated STAT1 is transferred to the nucleus and acts as a transcriptional factor to enhance interferon-stimulated genes replication [ISGs] [Walter MR 2020]. Also, Anti-PD-1 antibody activates STAT1 through IL-12 activation [Lu et al. 2109].

STAT1-Pt molecule selection to be included in a therapeutic approach after linking to nivolumab can be

an effective therapy. As illustrated above, PD-1/PD-L1 transcriptional cascade reactions by cancer cells share the same activators of STAT1. So, PD-L1 and PD-1 enhancement is resulted by malignant cells` mediators against nivolumab, helping STAT1-Pt transported to the nucleus [fig 1]. So endocytosis of the nivolumab-activated STAT1-Pt complex into the cancer cell will permit deposition of platinum loaded on STAT1 onto the Nucleus [in response to malignant cell defense], specifically PD-1 and PD-L1 gene promoter regions. Also that can be upregulated by the advantage of nivolumab that can be endocytosed more than other anti-PD1 antibodies [Ben Saad et al. 2020].



**Figure [1]:** Stages of nivolumab-STAT Pt endocytosis and functioning.[A] Endocytosis of the molecule into the malignant cell after binding to PD-1 receptor.[B] Nivolumab is dissociated from STAT1-Pt by the action of malignant cell glycosidase enzymes and releasing solamargine polymer [SM] free within the malignant cell cytoplasm. Malignant cells secrete IL-4, IL-13, CXCL8, TNF- $\alpha$  and IFN $\gamma$  for PD-1/PD-L1 genetic stimulation by various intracellular signals. [C] IL-4, IL-13, CXCL8, TNF- $\alpha$  and IFN $\gamma$  stimulate signaling pathways for elevation PD-1 surface proteins and simultaneously STAT1-Pt translocation to the malignant cell nucleus.[D] STAT1-Pt attaches to the malignant cell nucleus [PD-1 promoter region] the platinum molecules loaded on STAT1 making adducts with PD-1 promoter region adenine and guanine bases, so damaging the base of PD-1 gene.

#### c) Solamargine Specific Anti-Tumor Properties

Solamargine`s selective anti-cancer efficacy makes it a candidate for directing nivolumab specifically to malignant cells. Solamargine used in treatment of cancer cell lines of Ehrlich Carcinoma, Leukemia [K562], Colon Cancer [HT-29, HCT-15], Liver Cancer [HepG2, PLC/PRF/5, SMMC-7721], Lung Cancer [A549], Gastric Carcinoma [AGS], Pancreatic Carcinoma [MIA, PaCa-2], Renal Adenocarcinoma [786-0], Uterine Adenocarcinoma [HeLa 229], Ovarian Carcinoma

[JAM], Mesothelioma [NO36], Glioblastoma, Astrocytoma [U87-MG], Prostate Carcinoma [DV-145, LNCap, PC-3], Melanoma [A2058], Breast Cancer [T47D, MDA-MB-231], Osteosarcoma [U20S] and Squamous Cell Carcinoma [A431, SCC4, SCC9, SCC25][Bill, 2013].

Solamargine has multiple anti-cancer mechanisms such as stimulation the intrinsic and extrinsic pathways of apoptosis, increased function of external death receptors [TNFR-1, Fas receptor, TNFR-

1-associated death domain [TRADD], Fas-associated death domain [FADD], elevation of the intrinsic ratio of Bax to Bcl-2 and oncosis [Sun et al 2010].

Solamargine polymer will be the bridging molecule between nivolumab [after glycosylation its Fc portion] and STAT1-Pt complex. [Chemical and biochemical reactions will be discussed later] Nivolumab, like other immune checkpoint inhibitors, are related to exaggerate immune-related Side effects, such as colitis, hepatitis and skin disorders because of cross-reaction with healthy PD-1 presenting hematopoietic

cells [Dyck et al. 2017]. Those autoimmune adverse reactions can be avoided by solamargine, in other words, it will restrict nivolumab binding with normal PD-1 presenting cells and will facilitate selectivity towards PD-1 expressing malignant cells and TAMs only.

Solamargine molecular formula is  $C_{45}H_{73}NO_{15}$  with a mass of 868.04 Da. Its systematic name is [22R, 25R]- spiro-5-ene-3 $\beta$ -L- $\alpha$ -L-rhamnopyranosyl-[1 2glu]-O- $\alpha$ -yl-rhamnopyranosyl-[14glu]- $\beta$ -D-glucopyranose. [fig.2]

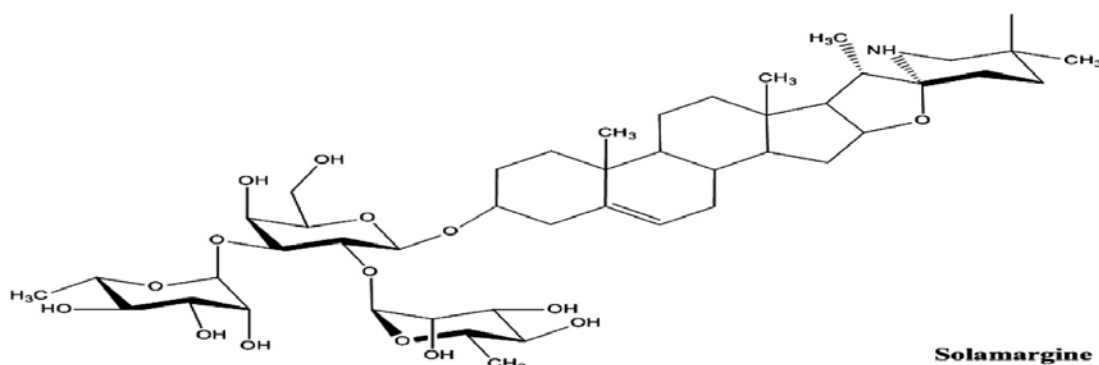


Figure [2]: Molecular formula of solamargine.

d) Structure of Nivolumab-Stat1- Platinum Molecule

The innovated molecule consists of nivolumab [anti PD-1 MAB] glycosylated with glucopyranose of solamargine. Solamargine  $\beta$ -solamargine polymer

through its Fc region with is bound to glycosylated cisplatin molecules loaded on seven lysine residues of biochemically activated synthesized STAT1.[Fig 3]

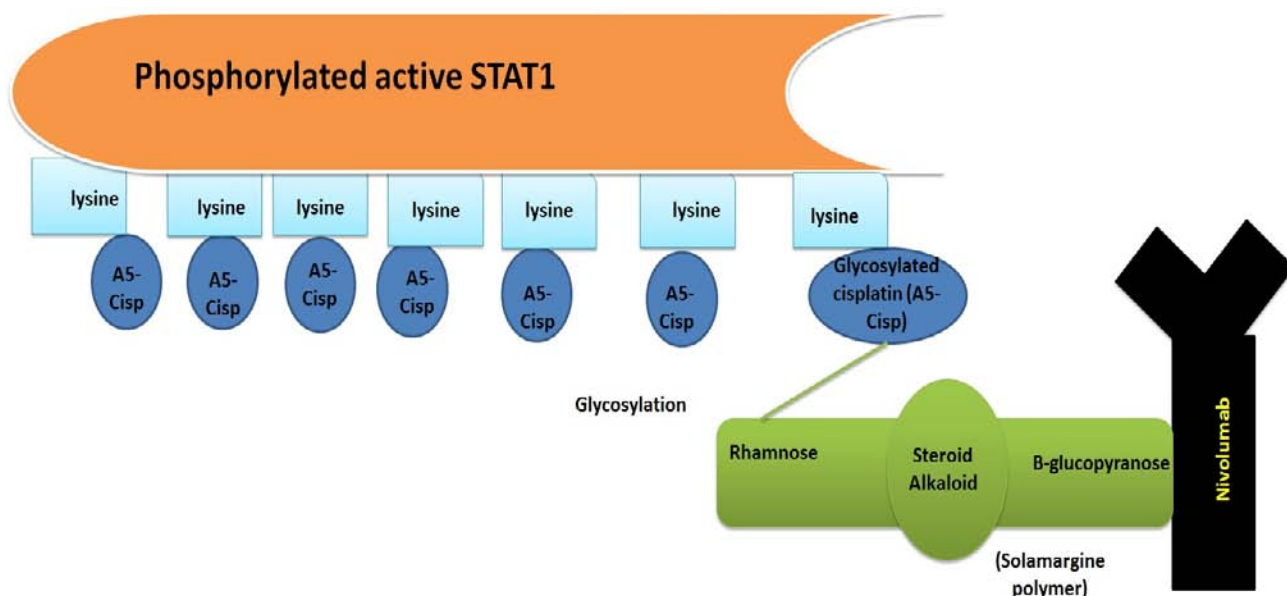


Figure [3]: Structure of nivolumab–STAT1 Pt molecule. Nivolumab Fc portion is glycosylated glucopyranose residue]. Glycosylated cisplatin molecules are  $\beta$ -with solamargine polymer [loaded on STAT1 lysine residues [reaction discussed later]. Glycosylated cisplatin is attached to solamargine polymer [rhamonse moiety] by rhamnosyl transferase.

#### e) *Nivolumab-Stat1 Pt Therapeutic Mechanisms*

On Nivolumab-STAT1 Pt administration, it runs within the body circulation towards PD-1 presenting malignant cells due to the presence of multiple targeting elements. The first is nivolumab's nature a monoclonal antibody [Anti-PD1] however, it can be directed towards PD-1 expressing hematopoietic cells such as CD4+ and CD8+ T cells, natural killer [NK] cells, natural killer T [NKT] cells, B cells, macrophages, and dendritic cells resulting in immunosuppression. Also, it can bind to hepatocytes, vascular endothelial cells, epithelial cells, myocytes, pancreatic islet cells, placenta and eye, initiating autoimmune adverse reactions. Here the role of solamargine polymer comes. Solamargine glycoside is considered as an attracting factor for cancer cell requirements for proliferation and spread. Also, it is characterized by selective tumor cell binding. The third is the activated STAT1 which is the needed transcriptional factor for malignant cells to overexpress PD-1 as a defending pathway against nivolumab. So cancer cells and TAMs will uptake the activated STAT1 because it is their rescue to escape from the immune system. After all, it is responsible for increasing PD-1 expression on their surface membranes.

Also, nivolumab-STAT1 Pt molecule is a concentrated anti-cancer therapeutic molecule. Nivolumab is a human immunoglobulin G4 PD-1 immune checkpoint inhibitor antibody that attenuates PD-1 interaction with PD-L1/PD-L2 receptors and stimulates anticancer immunity. It showed good therapeutic parameters [prolonged PFS and increased response rate] in the treatment of non-small-cell lung cancer [NSCLC], melanoma, renal cell carcinoma [RCC] and other cancers. [Guo et al. 2016]. Nivolumab [IgG4] Fc region consists of double heavy-chain C $\gamma$ 2 and C $\gamma$ 3 constant domains that are bound to two Fabs, comprising VH and C $\gamma$ 1 [heavy chain] and VL and Cj/k [light chain] domains, through a hinge. The Fc region has the dominant role for functioning. A biantennary oligosaccharide moiety, covalently attached to Asn297 in the Cc2 domain, contains two N-acetylglucosamine residues, and a branching mannose residue to which  $\alpha$ [1-3] and  $\alpha$ [1-6] 'arms' of mannose and N-acetylglucosamine residues are attached. The oligosaccharide moiety can additionally contain a fucose residue, attached to the first N-acetylglucosamine residue, and galactose and sialic acid residues attached to the  $\alpha$ [1-3] and  $\alpha$ [1-6] arms [Davies and Sutton 2015.].

Activated STAT1-Pt molecule will be a trap for malignant cells. Malignant cells use activated STAT1 for tumor spread and immunity resistance [Messi et al. 2017]. This is observed in the reduction of NK cellular activity in multiple myeloma, acute myeloid leukemia [AML], and acute lymphoblastic leukemia [ALL] [Bellucci et al. 2015] after IFN $\gamma$  stimulation. While in head and cancer, wild type of EGFR induces

JAK2/STAT1 activation that promotes the antitumor effect by PD-L1 over-transcription [Concha-Benavente et al. 2016] Interferon regulatory factor 1 [IRF1], which is a downstream activator of STAT1 just after IFN $\gamma$  stimulation, has an enhancing effect of PD-L1 genetic activity [Lee et al. 2006].

Activated STAT1 contains 2-acetyl serine which is essential for protein integrity [Bienvenut et al. 2012]. Lysine residues 114, 175, 29, 366, 525, 637 and 665 are methylated. Methylation gives the advantage of the antiviral function. Methylation is done by methyltransferase SETD2 [Chen et al. 2015]. Lysine residues without methylation are the target ones to be conjugated with glycosylated cisplatin molecules. That conjugation will not affect STAT1 function in malignancy. Glutamic acid residues 657, 705 are ADP-ribosylated by PARP 14. Glutamic acid ADP ribosylation suppresses STAT1 phosphorylation [Iwata et al 2016]. During synthesis and purification of activated STAT1-Pt molecule, ADP ribosylation of glutamic acid will be avoided. Tyrosine 701 residue is phosphorylated in response to Janus protein-tyrosine kinase and epidermal growth factor receptor stimulation after IFN $\gamma$  induction [Quelle et al. 1995, Iwata et al 2016]. Tyrosine 701 phosphorylation also is one by KIT-Asp [816] mutants in neoplastic mast cell lines [Chaix et al. 2011]. Serine residues 708, 745 phosphorylation occurs through IFN- $\alpha/\beta$  induction by IKK $\epsilon$ , so serine residues 708,745 phosphorylation is essential for STAT1 activation [Perwitasari et al. 2011]. Serine 727 phosphorylation and tyrosine 701 phosphorylation is really necessary for STAT1 activation. Serine 727 phosphorylation occurs by the action of etoposide and PKCdelta. [Brodie and Blumberg 2003, Wen et al. 1995]

Glycosylated cisplatin are combined with purified active STAT1 lysine residues. Glycosylated cisplatin [platinum IV] are prodrugs that undergo activation to platinum II by malignant cell reductants such as ascorbic acid and glutathione. Being a prodrug and activation inside malignant cells only minimize possible side effects to a significant extent. Glycosylation helps attachment to lysine residues and at the same time, glycosylation to rhamnose residues of solamargine [fig.2]. Also, platinum IV drugs are favored other than platinum II ones because they are more stable and have longer half-life than platinum II drugs. Glycosylation adjusts steric hindrance and length to enable cisplatin for a reduction potential and positive shift to the cancer cells. It is not forgettable that platinum IV drugs have lipophilicity more than platinum II. Lipophilicity permits more access of platinum IV drugs for tumoral cellular uptake and DNA adenine-guanine platination. The used platinum IV drug in STAT1-Pt is A5 complex of cisplatin [fig. 4]. It is known that A5 has more efficacy towards HeLa, A549, MCF-7 and PC3 cancer cell lines other than cisplatin and oxaliplatin. [Jing, et al., 2016].



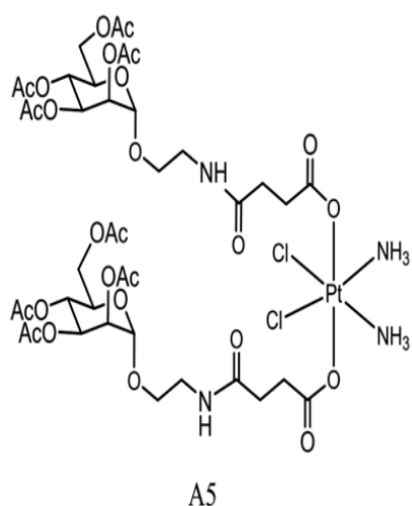
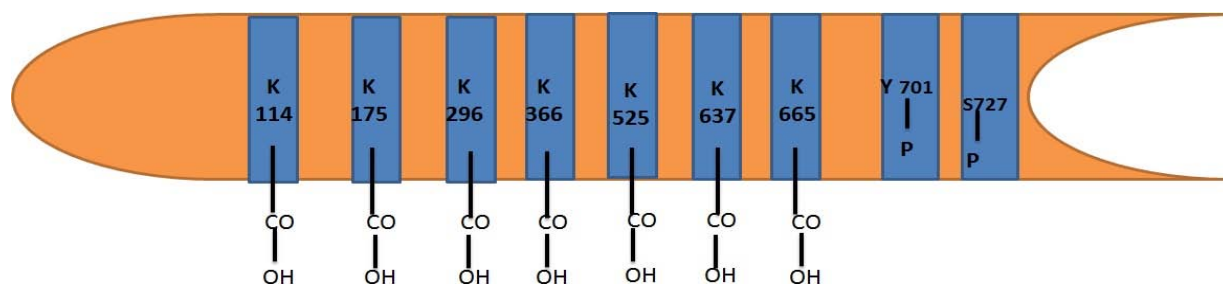


Figure [4]: Structure of A5 cisplatin complexes.

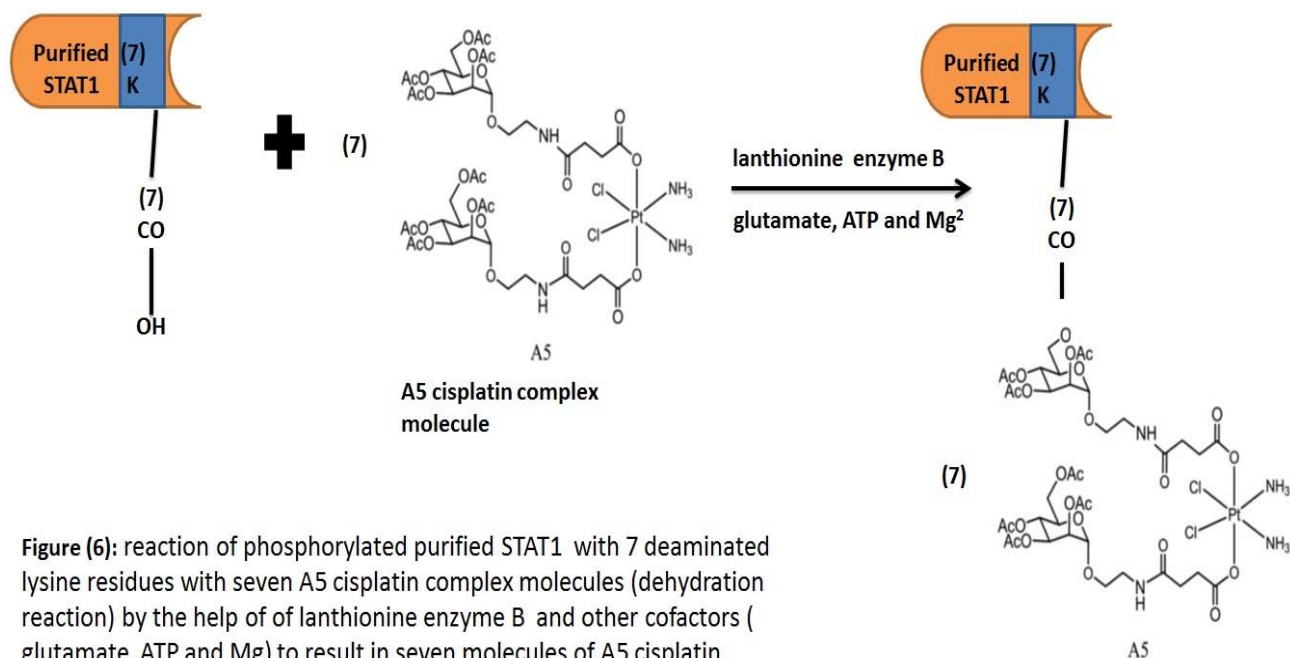
- f) *Chemical and Biochemical Steps for Nivolumab-Stat1-Platinum Molecule*

  1. *Purification of activated STAT1:* HeLa cells have a major role in STAT1 cultivation. HeLa cells will be incorporated with Lysine 6- dehydrogenase gene delivered by exosome pDNA [plasmid DNA] containing the enzyme gene [Munagala et al. 2021], then keeping PH 10.1 and temperature 70 o C [Heydari et al. 2004]. 70°C will not affect STAT1 protein integrity because its denaturation temperature is 95°C [Sisler et al. 2015]. The resulted STAT1 within HeLa cells will be unphosphorylated and deaminated lysine residues. Then IFN stimulation of HeLa cells for tyrosine phosphorylation. [Kim and Maniatis 1996]. However, it is inactivated in the nucleus by unknown tyrosine phosphatase PTP and purified as Stat1-PTP from HeLa nuclear extract. [ten Hoeve et al. 2002] Then E6-E6AP complex [one of the Human Papilloma Virus E6 oncoproteins] can be used to combine with Stat1-PTP and degrade it in vitro to yield purified phosphorylated STAT1 [Jing et al. 2007]. The purified STAT1 is phosphorylated at its tyrosine 701 and serine 727 because of IFN stimulation besides deaminated lysine residues 114, 175, 296, 366, 525, 637 and 665, and that is the wanted form to be used in our molecule [fig.5].
  2. A5 complex molecules of cisplatin will be reacted with deaminated Lysine residues of purified STAT1 [not methylated] by lanthionine biosynthetic enzyme B [LanB] proteins in the presence of glutamate, ATP and Mg2+. [Garg et al. 2103]. This in vitro dehydration reaction between the glycosidic component of 7 molecules of A5 complex cisplatin and 7deaminated lysine residues of phosphorylated STAT1 to result in STAT1-A5 cisplatin molecule. [fig.6]
  3. Production of glycosylated Nivolumab with solamargine polymer: Transgenic immunization for human immunoglobulin loci with genetically recombinant Chinese hamster ovarian cells expressing human PD-1/PD-L1/human IgG1 Fc fusion protein. [Mimura et al. 2018]. The core complex biantennary heptasaccharide attached to the purified nivolumab Fc region is GlcNAc2Man3GlcNAc2. Previously mentioned heptasacchride can be attached to G0, G1 or G2 saccharide according to the number of galactose residues. G0 has no galactose residue, and G1 has one galactose terminal, while G2 has two galactose residues. [Mimura et al. 2018]. 6-glucosyl-transferase enzyme [CaUGT3] can elongate the heptasacchride G0/G1/G2 of nivolumab as a sugar acceptor to β-D-glucopyranose of solamargine. [Masada et al. 2009]. The enzymatic assay is used with the purified nivolumab using quercetin 3-O-glucoside as an acceptor substrate in the presence of UDP-glucose. The same retention time and UV absorption of quercetin 3-O-gentiobioside result in nivolumab [one side Fc region] with solamargine polymer [Masada et al. 2009]. [fig.7]
  4. Solamargine rhamnose moiety is transferred to one A5 complex molecule of cisplatin [attached to STAT1] by rhamnosyltransferases besides the nucleotide diphosphate-sugar UDP-rhamnose [UDP-Rha] as a substrate to result in STAT1-Pt-Nivolumab molecule [Lairson et al. 2008] [fig.3].

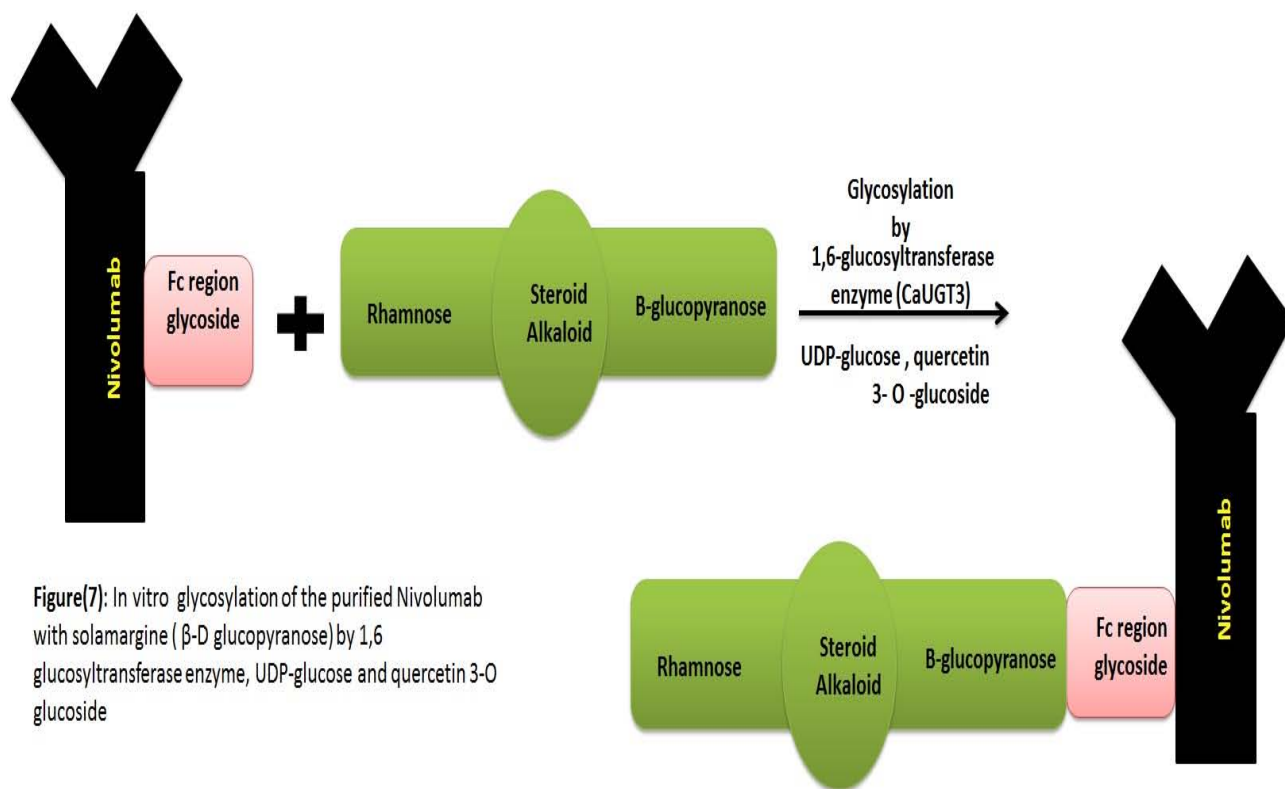




**Figure (5):** Structure of purified STAT1 that contains phosphorylated tyrosine 701, phosphorylated serine 727 and seven (114, 175, 296, 366, 525, 637, 665) deaminated lysine residues



**Figure (6):** reaction of phosphorylated purified STAT1 with 7 deaminated lysine residues with seven A5 cisplatin complex molecules (dehydration reaction) by the help of of lanthionine enzyme B and other cofactors ( glutamate, ATP and Mg) to result in seven molecules of A5 cisplatin complex attached to seven deaminated lysine residues on one STAT1 molecule.



**Figure(7):** In vitro glycosylation of the purified Nivolumab with solamargine ( $\beta$ -D glucopyranose) by 1,6 glucosyltransferase enzyme, UDP-glucose and quercetin 3-O glucoside

## II. CONCLUSION

STAT1-Pt nivolumab molecules are targeted towards malignant cells only by the bridging solamargine glycoside. After binding nivolumab to PD-1 expressing malignant cell, endocytosis occurs. Here, glycosidic bonds of solamargine - nivolumab and solamargine- A5 cisplatin STAT1 are hydrolyzed by malignant cell glycosidase enzymes. STAT1- Pt [A5 cisplatin molecules] are transported to the nucleus and seven molecules of platinum IV of A5 cisplatin complex molecule are reduced by malignant cell reductants [glutathione and ascorbic acid] to functioning cytotoxic platinum II. The active form of the used STAT1 in the therapeutic molecule is essential because it does facilitate its nuclear translocation upon different cytokines and IFN- $\gamma$  secretion by malignant cells [malignant cells use those mediators for over recruitment PD-1/PD-L1 genes to resist nivolumab]. So the more mediators secretion, the more STAT1-A5 cisplatin movement to the malignant cell nucleus. In the end, the aim of the molecule is reached, which is damaging PD-1/PD-L1 genetic promoter regions by multiple concentrated platinum containing STAT1 molecules. Also anti-tumor role of endocytosed solamargine is not forgotten as it becomes free after glycosidic bonds hydrolysis. While nivolumab exerts anti PD-1 signaling pathway, it can be considered a targeting molecule besides solamargine towards PD-1 expressing malignant cell for initiating the cytotoxic reactions of the innovated therapeutic molecule.

## Conflict of Interest

Authors have no conflict of interest.

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#### Abbreviations

STAT1: Signal transducer and activator of transcription 1.  
 PD-1: Programmed death 1  
 PD-L1: Programmed death ligand 1  
 NK: natural killer  
 NKT: Natural killer T cells  
 DC: Dendritic cells  
 TGF- $\beta$ : cytokines transforming growth factor- $\beta$   
 IL-10: interleukin-10  
 TAMs: tumor associated macrophages  
 JAKs: Janus Kinases  
 IFN- $\gamma$ : interferon  $\gamma$   
 ISGs: interferon stimulated genes

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**8. Make every effort:** Make every effort to mention what you are going to write in your paper. That means always have a good start. Try to mention everything in the introduction—what is the need for a particular research paper. Polish your work with good writing skills and always give an evaluator what he wants. Make backups: When you are going to do any important thing like making a research paper, you should always have backup copies of it either on your computer or on paper. This protects you from losing any portion of your important data.

**9. Produce good diagrams of your own:** Always try to include good charts or diagrams in your paper to improve quality. Using several unnecessary diagrams will degrade the quality of your paper by creating a hodgepodge. So always try to include diagrams which were made by you to improve the readability of your paper. Use of direct quotes: When you do research relevant to literature, history, or current affairs, then use of quotes becomes essential, but if the study is relevant to science, use of quotes is not preferable.

**10. Use proper verb tense:** Use proper verb tenses in your paper. Use past tense to present those events that have happened. Use present tense to indicate events that are going on. Use future tense to indicate events that will happen in the future. Use of wrong tenses will confuse the evaluator. Avoid sentences that are incomplete.

**11. Pick a good study spot:** Always try to pick a spot for your research which is quiet. Not every spot is good for studying.

**12. Know what you know:** Always try to know what you know by making objectives, otherwise you will be confused and unable to achieve your target.

**13. Use good grammar:** Always use good grammar and words that will have a positive impact on the evaluator; use of good vocabulary does not mean using tough words which the evaluator has to find in a dictionary. Do not fragment sentences. Eliminate one-word sentences. Do not ever use a big word when a smaller one would suffice.

Verbs have to be in agreement with their subjects. In a research paper, do not start sentences with conjunctions or finish them with prepositions. When writing formally, it is advisable to never split an infinitive because someone will (wrongly) complain. Avoid clichés like a disease. Always shun irritating alliteration. Use language which is simple and straightforward. Put together a neat summary.

**14. Arrangement of information:** Each section of the main body should start with an opening sentence, and there should be a changeover at the end of the section. Give only valid and powerful arguments for your topic. You may also maintain your arguments with records.

**15. Never start at the last minute:** Always allow enough time for research work. Leaving everything to the last minute will degrade your paper and spoil your work.

**16. Multitasking in research is not good:** Doing several things at the same time is a bad habit in the case of research activity. Research is an area where everything has a particular time slot. Divide your research work into parts, and do a particular part in a particular time slot.

**17. Never copy others' work:** Never copy others' work and give it your name because if the evaluator has seen it anywhere, you will be in trouble. Take proper rest and food: No matter how many hours you spend on your research activity, if you are not taking care of your health, then all your efforts will have been in vain. For quality research, take proper rest and food.

**18. Go to seminars:** Attend seminars if the topic is relevant to your research area. Utilize all your resources.

**19. Refresh your mind after intervals:** Try to give your mind a rest by listening to soft music or sleeping in intervals. This will also improve your memory. Acquire colleagues: Always try to acquire colleagues. No matter how sharp you are, if you acquire colleagues, they can give you ideas which will be helpful to your research.





**20. Think technically:** Always think technically. If anything happens, search for its reasons, benefits, and demerits. Think and then print: When you go to print your paper, check that tables are not split, headings are not detached from their descriptions, and page sequence is maintained.

**21. Adding unnecessary information:** Do not add unnecessary information like "I have used MS Excel to draw graphs." Irrelevant and inappropriate material is superfluous. Foreign terminology and phrases are not apropos. One should never take a broad view. Analogy is like feathers on a snake. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Never oversimplify: When adding material to your research paper, never go for oversimplification; this will definitely irritate the evaluator. Be specific. Never use rhythmic redundancies. Contractions shouldn't be used in a research paper. Comparisons are as terrible as clichés. Give up ampersands, abbreviations, and so on. Remove commas that are not necessary. Parenthetical words should be between brackets or commas. Understatement is always the best way to put forward earth-shaking thoughts. Give a detailed literary review.

**22. Report concluded results:** Use concluded results. From raw data, filter the results, and then conclude your studies based on measurements and observations taken. An appropriate number of decimal places should be used. Parenthetical remarks are prohibited here. Proofread carefully at the final stage. At the end, give an outline to your arguments. Spot perspectives of further study of the subject. Justify your conclusion at the bottom sufficiently, which will probably include examples.

**23. Upon conclusion:** Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium through which your research is going to be in print for the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects of your research.

## INFORMAL GUIDELINES OF RESEARCH PAPER WRITING

### Key points to remember:

- Submit all work in its final form.
- Write your paper in the form which is presented in the guidelines using the template.
- Please note the criteria peer reviewers will use for grading the final paper.

### Final points:

One purpose of organizing a research paper is to let people interpret your efforts selectively. The journal requires the following sections, submitted in the order listed, with each section starting on a new page:

*The introduction:* This will be compiled from reference matter and reflect the design processes or outline of basis that directed you to make a study. As you carry out the process of study, the method and process section will be constructed like that. The results segment will show related statistics in nearly sequential order and direct reviewers to similar intellectual paths throughout the data that you gathered to carry out your study.

### The discussion section:

This will provide understanding of the data and projections as to the implications of the results. The use of good quality references throughout the paper will give the effort trustworthiness by representing an alertness to prior workings.

Writing a research paper is not an easy job, no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record-keeping are the only means to make straightforward progression.

### General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

**To make a paper clear:** Adhere to recommended page limits.



### *Mistakes to avoid:*

- Insertion of a title at the foot of a page with subsequent text on the next page.
- Separating a table, chart, or figure—confine each to a single page.
- Submitting a manuscript with pages out of sequence.
- In every section of your document, use standard writing style, including articles ("a" and "the").
- Keep paying attention to the topic of the paper.
- Use paragraphs to split each significant point (excluding the abstract).
- Align the primary line of each section.
- Present your points in sound order.
- Use present tense to report well-accepted matters.
- Use past tense to describe specific results.
- Do not use familiar wording; don't address the reviewer directly. Don't use slang or superlatives.
- Avoid use of extra pictures—include only those figures essential to presenting results.

### **Title page:**

Choose a revealing title. It should be short and include the name(s) and address(es) of all authors. It should not have acronyms or abbreviations or exceed two printed lines.

**Abstract:** This summary should be two hundred words or less. It should clearly and briefly explain the key findings reported in the manuscript and must have precise statistics. It should not have acronyms or abbreviations. It should be logical in itself. Do not cite references at this point.

An abstract is a brief, distinct paragraph summary of finished work or work in development. In a minute or less, a reviewer can be taught the foundation behind the study, common approaches to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Use comprehensive sentences, and do not sacrifice readability for brevity; you can maintain it succinctly by phrasing sentences so that they provide more than a lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study with the subsequent elements in any summary. Try to limit the initial two items to no more than one line each.

*Reason for writing the article—theory, overall issue, purpose.*

- Fundamental goal.
- To-the-point depiction of the research.
- Consequences, including definite statistics—if the consequences are quantitative in nature, account for this; results of any numerical analysis should be reported. Significant conclusions or questions that emerge from the research.

### **Approach:**

- Single section and succinct.
- An outline of the job done is always written in past tense.
- Concentrate on shortening results—limit background information to a verdict or two.
- Exact spelling, clarity of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else.

### **Introduction:**

The introduction should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable of comprehending and calculating the purpose of your study without having to refer to other works. The basis for the study should be offered. Give the most important references, but avoid making a comprehensive appraisal of the topic. Describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will give no attention to your results. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here.



*The following approach can create a valuable beginning:*

- Explain the value (significance) of the study.
- Defend the model—why did you employ this particular system or method? What is its compensation? Remark upon its appropriateness from an abstract point of view as well as pointing out sensible reasons for using it.
- Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
- Briefly explain the study's tentative purpose and how it meets the declared objectives.

#### **Approach:**

Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done. Sort out your thoughts; manufacture one key point for every section. If you make the four points listed above, you will need at least four paragraphs. Present surrounding information only when it is necessary to support a situation. The reviewer does not desire to read everything you know about a topic. Shape the theory specifically—do not take a broad view.

As always, give awareness to spelling, simplicity, and correctness of sentences and phrases.

#### **Procedures (methods and materials):**

This part is supposed to be the easiest to carve if you have good skills. A soundly written procedures segment allows a capable scientist to replicate your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order, but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt to give the least amount of information that would permit another capable scientist to replicate your outcome, but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section.

When a technique is used that has been well-described in another section, mention the specific item describing the way, but draw the basic principle while stating the situation. The purpose is to show all particular resources and broad procedures so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step-by-step report of the whole thing you did, nor is a methods section a set of orders.

#### **Materials:**

*Materials may be reported in part of a section or else they may be recognized along with your measures.*

#### **Methods:**

- Report the method and not the particulars of each process that engaged the same methodology.
- Describe the method entirely.
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- Simplify—detail how procedures were completed, not how they were performed on a particular day.
- If well-known procedures were used, account for the procedure by name, possibly with a reference, and that's all.

#### **Approach:**

It is embarrassing to use vigorous voice when documenting methods without using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result, when writing up the methods, most authors use third person passive voice.

Use standard style in this and every other part of the paper—avoid familiar lists, and use full sentences.

#### **What to keep away from:**

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings—save it for the argument.
- Leave out information that is immaterial to a third party.



**Results:**

The principle of a results segment is to present and demonstrate your conclusion. Create this part as entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Use statistics and tables, if suitable, to present consequences most efficiently.

You must clearly differentiate material which would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matters should not be submitted at all except if requested by the instructor.

**Content:**

- Sum up your conclusions in text and demonstrate them, if suitable, with figures and tables.
- In the manuscript, explain each of your consequences, and point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation of an exacting study.
- Explain results of control experiments and give remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or manuscript.

**What to stay away from:**

- Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- Do not include raw data or intermediate calculations in a research manuscript.
- Do not present similar data more than once.
- A manuscript should complement any figures or tables, not duplicate information.
- Never confuse figures with tables—there is a difference.

**Approach:**

As always, use past tense when you submit your results, and put the whole thing in a reasonable order.

Put figures and tables, appropriately numbered, in order at the end of the report.

If you desire, you may place your figures and tables properly within the text of your results section.

**Figures and tables:**

If you put figures and tables at the end of some details, make certain that they are visibly distinguished from any attached appendix materials, such as raw facts. Whatever the position, each table must be titled, numbered one after the other, and include a heading. All figures and tables must be divided from the text.

**Discussion:**

The discussion is expected to be the trickiest segment to write. A lot of papers submitted to the journal are discarded based on problems with the discussion. There is no rule for how long an argument should be.

Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implications of the study. The purpose here is to offer an understanding of your results and support all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of results should be fully described.

Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact, you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved the prospect, and let it drop at that. Make a decision as to whether each premise is supported or discarded or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."



Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work.

- You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- Give details of all of your remarks as much as possible, focusing on mechanisms.
- Make a decision as to whether the tentative design sufficiently addressed the theory and whether or not it was correctly restricted. Try to present substitute explanations if they are sensible alternatives.
- One piece of research will not counter an overall question, so maintain the large picture in mind. Where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

#### **Approach:**

When you refer to information, differentiate data generated by your own studies from other available information. Present work done by specific persons (including you) in past tense.

Describe generally acknowledged facts and main beliefs in present tense.

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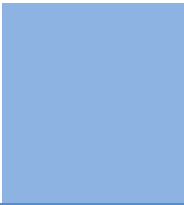


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	A-B	C-D	E-F
<i>Abstract</i>	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
<i>Introduction</i>	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
<i>Methods and Procedures</i>	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
<i>Result</i>	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
<i>Discussion</i>	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
<i>References</i>	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring





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