Scientific and Manufacturing Company «ECOPHARM» LTD.,

Ukraine

CIIE , Shanghai , November 05-10,2019

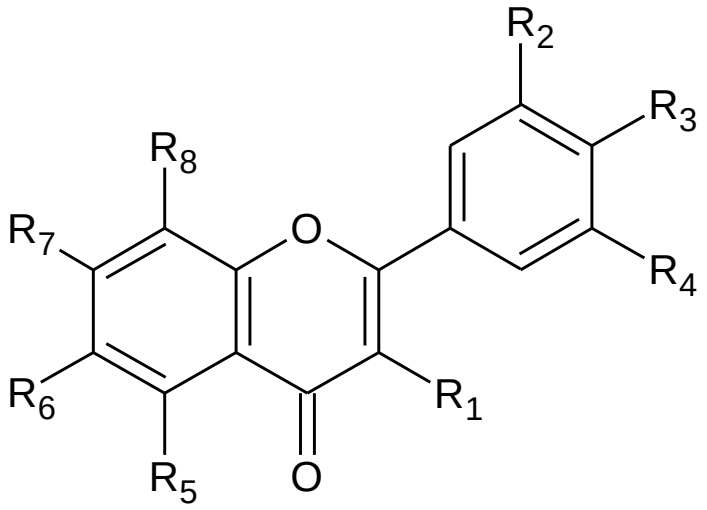
RESULTS OF PRECLINICAL AND CLINICAL STUDIES OF ACTIVE SUBSTANCE PROTEFLAZID (API)

Proteflazidum® contains flavonoids, natural-occurring compounds isolated from wild gramineous plants Deschampsia caespitosa L. and Calamagrostis epigeios L. The composition was developed by the Ukrainian research team under the auspices of S&MCo.” Ecopharm” Ltd.

Deschampsia caespitosa L., tussock grass

Cal*amagr*ostis epigeios L., reed grass

MOLECULAR STRUCTURE OF PROTEFLAZID API



Tricin: R_3,R_5, R_7 = OH; R_2, R_4 = OMe; R_1,R_6, R_8 = H

Tricin glycosides: R_1 = H; R_2, R_4 = OMe; R_3, R_5, R_7 = O-glycoside or OH; R_6, R_8 = H or C-glycoside

Luteolin: R_2, R_3, R_5, R_7 = OH; R_1, R_4, R_6, R_8 = H

Luteolin glycosides: R_1 = H; R_2, R_3, R_5, R_7 = O-glycoside or OH; R_4, R_6, R_8 = H or C-glycoside

Apigenin: R_3, R_5, R_7 = OH; R_1, R_2, R_4,R_6, R_8 = H

Apigenin glycosides: R_1 = H; R_3,R_5, R_7 = O-glycoside or OH;R_2, R_4, R_6, R_8 = H or C- glycoside

CHEMICAL STRUCTURE OF PROTEFLAZID API

- **Composition**
- ***Active substances of Proteflazid*** represent complex compounds of flavonoids and their glycosides:
- [Tricin, Tricin–7–O or 8–C glycoside] : [Luteolin 7-O or 8–C glycoside] : [Apigenin, Apigenin–7-O or 8–C glycoside] = (3 : 4 : 1) amounting to 7.4–8.5% mass;
- *Other natural substances*:
- - related substances – glycosides of quercetin and rhamnazin up to 0.07% mass;
- - amino acids (4.5 – 5.5) % mass;
- - carbohydrates and carboxylic acids (7.0 – 8,0) % mass.;
- - natural polymers (polysaccharides, pectins, chlorophyll a and b, hemicellulose A and B) – the rest;
- Mass of dry residue of the composition 0.7 – 2.0% mass;

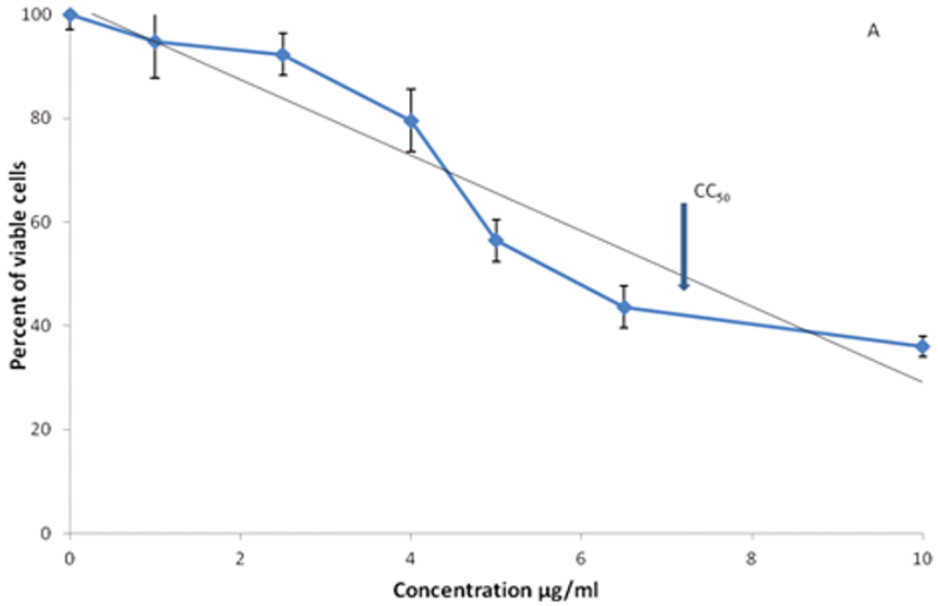
ACTIVE FLAVONOIDS SUBSTANCES OF PROTEFLAZID

Active flavonoids substances *of Proteflazid* represent complex compounds of flavonoids and their glycosides:

[Tricin, Tricin–7–O or 8–C glycoside] : [Luteolin 7-O or 8–C glycoside] : [Apigenin, Apigenin–7-O or 8–C glycoside] = (3 : 4 : 1)

SAFETY OF PROTEFLAZID AND MAJOR PHARMACOLOGICAL PARAMETERS OF ITS EFFICACY

- The results of MTT assay show dose-dependent cytotoxicity of Proteflazid with CC_{50} being of 6.2 µg/ml or about 9 µM assuming the average molecular weight of the major flavonoid compounds of the extract.

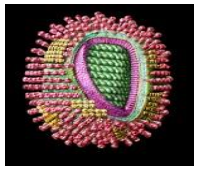


- Mutagenicity of Proteflazid in bone marrow cells of mice as well as in primary cultures of human embryonic tissue was not revealed (absence of pathological mitoses and chromatid aberrations).

VIRUS MODELS

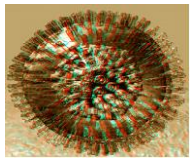
Influenza Virus

Study of the *in vitro* anti-influenza activity in MDCK cells and *in vivo* in mice with influenza virus induced pneumonia



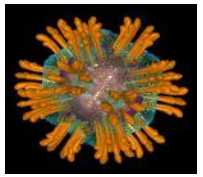
Herpes Virus (HSV)

Study of the *in vitro* antiherpetic activity in Vero cells infected with HSV types 1,2



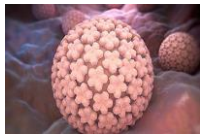
Hepatitis C Virus (HCV)

The surrogate model of hepatitis C virus- VBVD and HCV transfection model



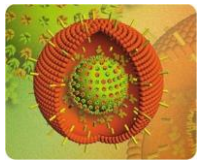
Human Papillomavirus (HPV)

Study *in vitro* in MT-4 cells of HPV transfection model



Epstein –Barr Virus (EBV)

Study *in vitro* in Raji cells from productive culture cell B95-8

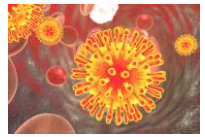


Adenovirus type 5 (ADV)

Study *in vitro* in Hep-2 cells

Human immunodeficiency virus type 1 (HIV -1)

Study *in vitro* in MT-4 cells



SUMMARY OF PROTEFLAZID ANTIVIRAL ACTIVITY *IN VITRO*

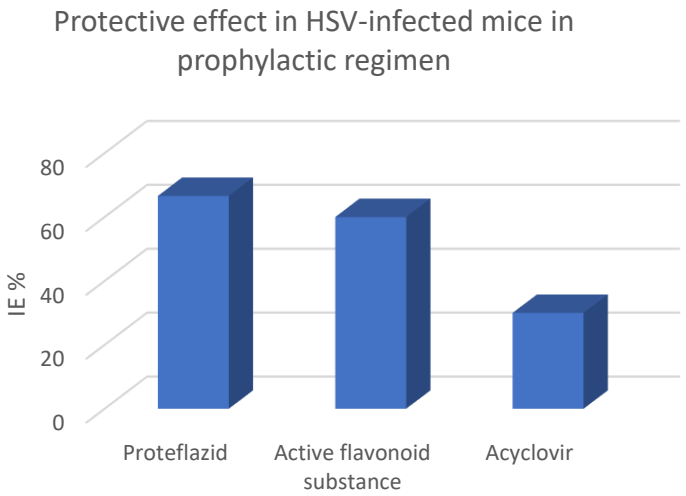
	Influenza virus (H1N1). MDBK cells			Herpes (HSV-1), RK13 cells			HCV, MT-4 cells			RNA polymerase T7, IC_{50}, µg/ml
	CC_{50} µg/ml	EC_{50} µg/ml	Thera-peutic index	CC_{50} µg/ml	EC_{50} µg/ml	Thera-peutic index	CC_{50} µg/ml	EC_{50} µg/ml	Thera-peutic index	
Proteflazid	7	0.045	155.6	6.8	0.052	130	6.8	0.01	680	0.07
Active flavonoids substance	240	1.5	160	240	0.375	640	240	2.4	100	4

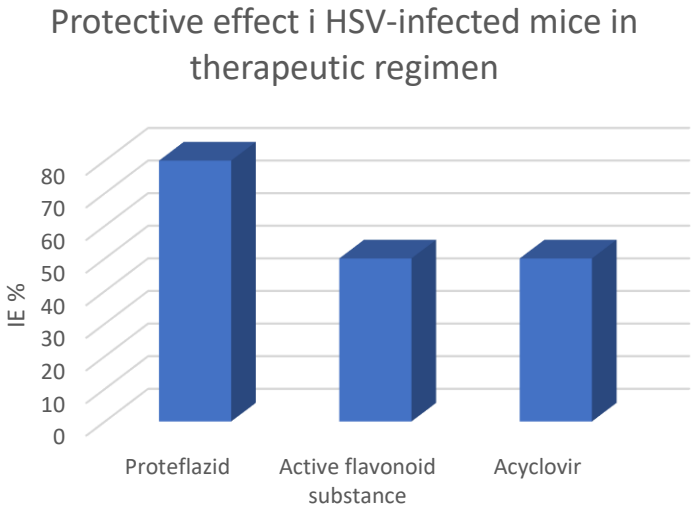
	HPV (human papilloma virus) MT-4 cells			Epstein-Barr virus Raji cells			Adenovirus Hep-2 cells			HIV MT-4 cells		
	CC_{50} µg/ml	EC_{50} µg/ml	Thera-peutic index	CC_{50} µg/ml	EC_{50} µg/ml	Thera-peutic index	CC_{50} µg/ml	EC_{50} µg/ml	Thera-peutic index	CC_{50} µg/ml	EC_{50} µg/ml	Thera-peutic index
Proteflazid	7	0.04	175	40	0.1	400	6.4	0.2	32	6.4	0.32	20
Active flavonoids substance	240	1.0	240	150	0.1	1500	-	-	-	-	-	-

PROTEFLAZID ANTI-INFLUENZA ACTIVITY *IN VIVO*

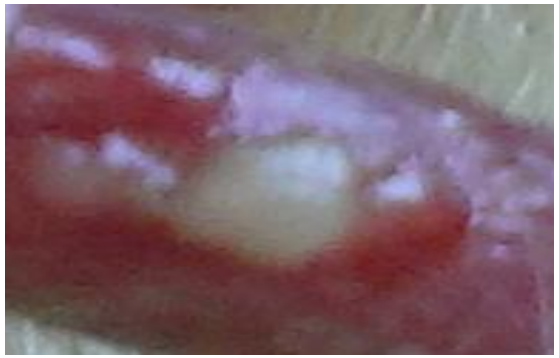
	Dose, µg/ml	IE,%
Prophylactic regimen		
Proteflazid	4.8	90
Active flavonoid substance	4.8	90
Therapeutic regimen		
Proteflazid	0.96	75
Active flavonoid substance	0.96	75

PROTEFLAZID HSV ACTIVITY *IN VIVO*



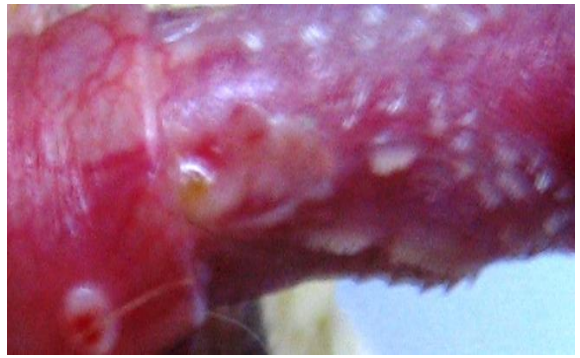


EFFICIENCY OF PROTEFLAZID IN GENITAL HERPES IN GUINEA PIGS



Genital herpes: swelling, hyperemia, rash

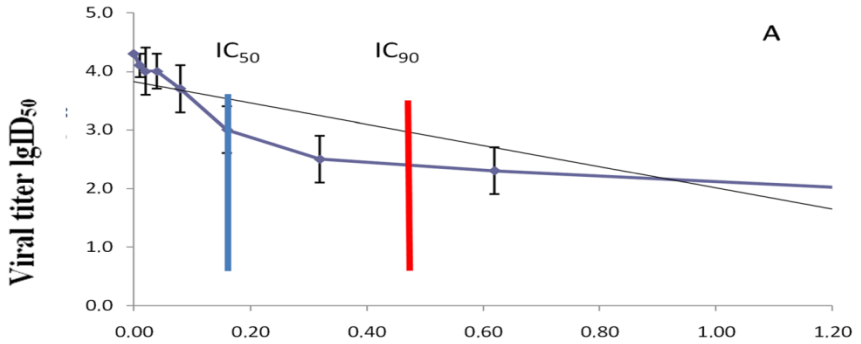
- Criteria for assessment of the severity of infectious process: area and grade of specific lesions, swelling, hyperemia, rash, discharges
- Maximal score for each feature 4 points

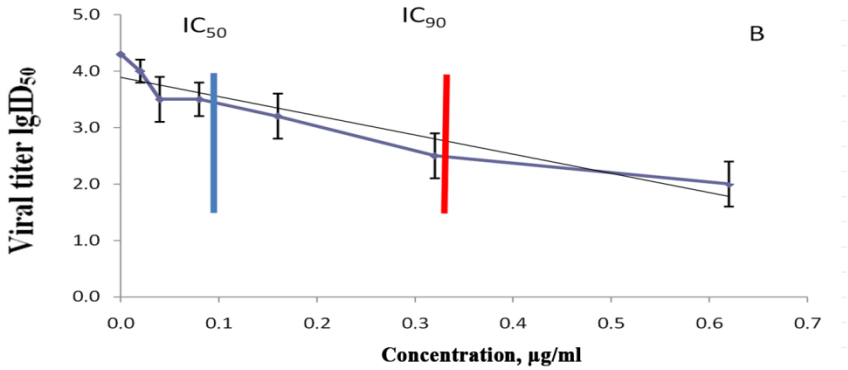


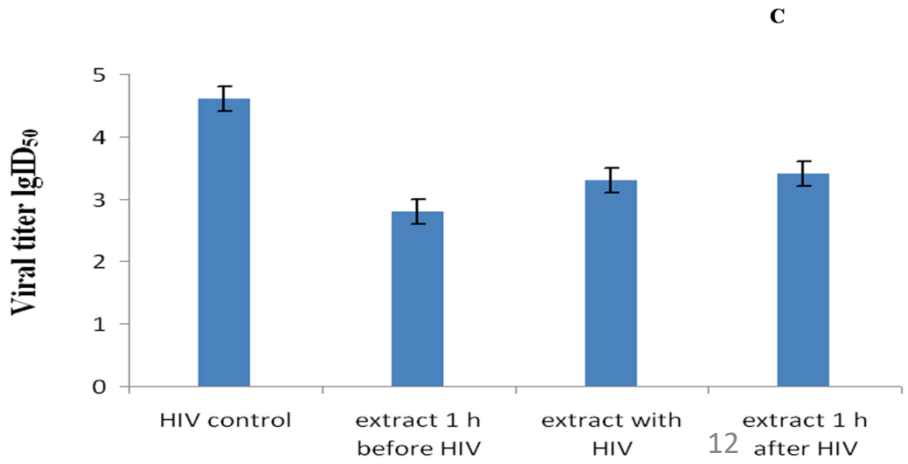
Treatment group Dose, mg	Duration of disease	p	Score for severity of pathological lesions, total points	Index of therapeutic activity, %
HSV without treatment	15 ± 3.2		51.0	
Acyclovir	9.75 ± 2.86	<0.05	22.0	56
Proteflazid 1.36	9.0 ± 1.39	<0.05	20.2	60
Proteflazid 6.8	5.0 ± 0.5	<0.05	5.0	95

REDUCTION OF HIV INFECTIOUS TITER IN MT-4 CELLS BY PROTEFLAZID

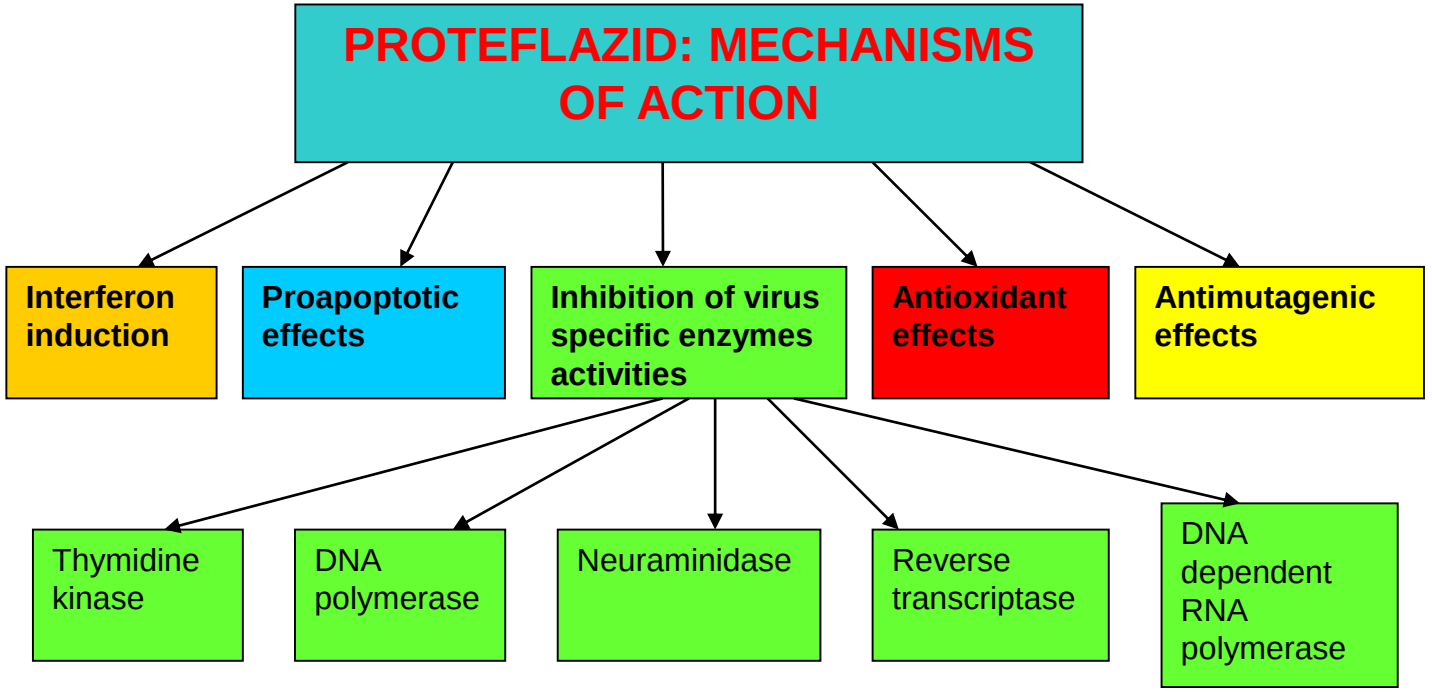
The two-fold serial dilutions of Proteflazid (A) or active flavonoids substance (B) were applied to each well with MT-4 cells simultaneously with 100 ID_{50} of HIV (each dilution of the extract in triplicate). The cells with virus and dilutions of the extract as well as controls were incubated for 5 days at 37 °C. Upon the incubation, HIV titer in each well was assayed by titration of the culture medium in MT4 cells. The decrease in HIV infectious titer was assessed. IC_{50} and IC_{90} were calculated by linear regression. In (C), the reduction of HIV infectious titer was assessed varying the sequence of HIV and extract application. The extract was applied at a dose of 0.32 µg/ml 1 hour before HIV infection, simultaneously with virus or 1 h following HIV infection (100 ID_{50} per well) in triplicates. The cells were incubated for 5 days at 37 °C. Then HIV titer in each well was assayed by titration of the HIV-containing medium in MT4 cells



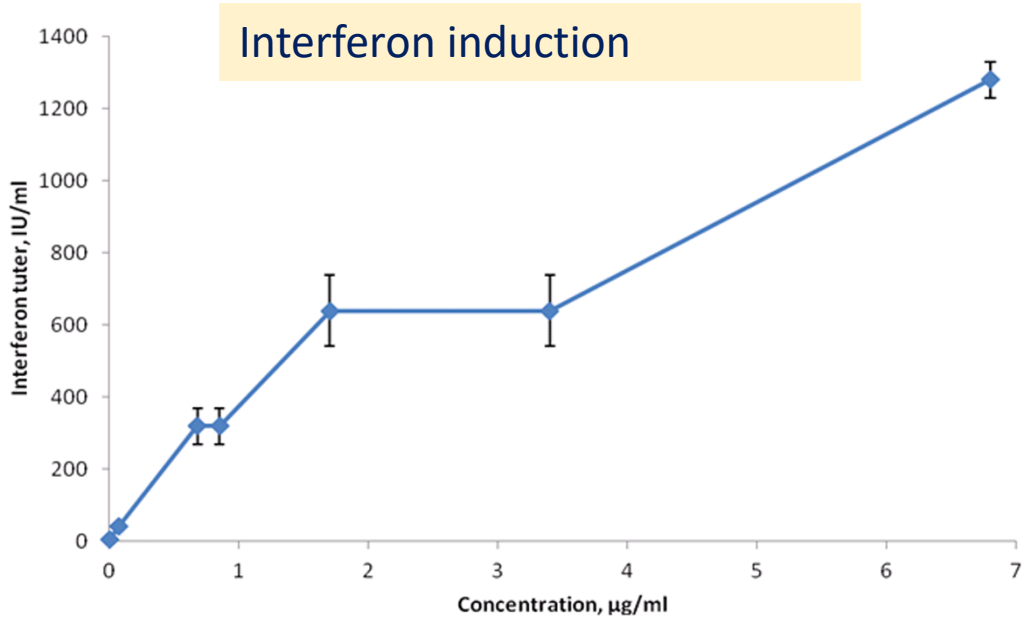




PROTEFLAZID: MECHANISMS OF ACTION



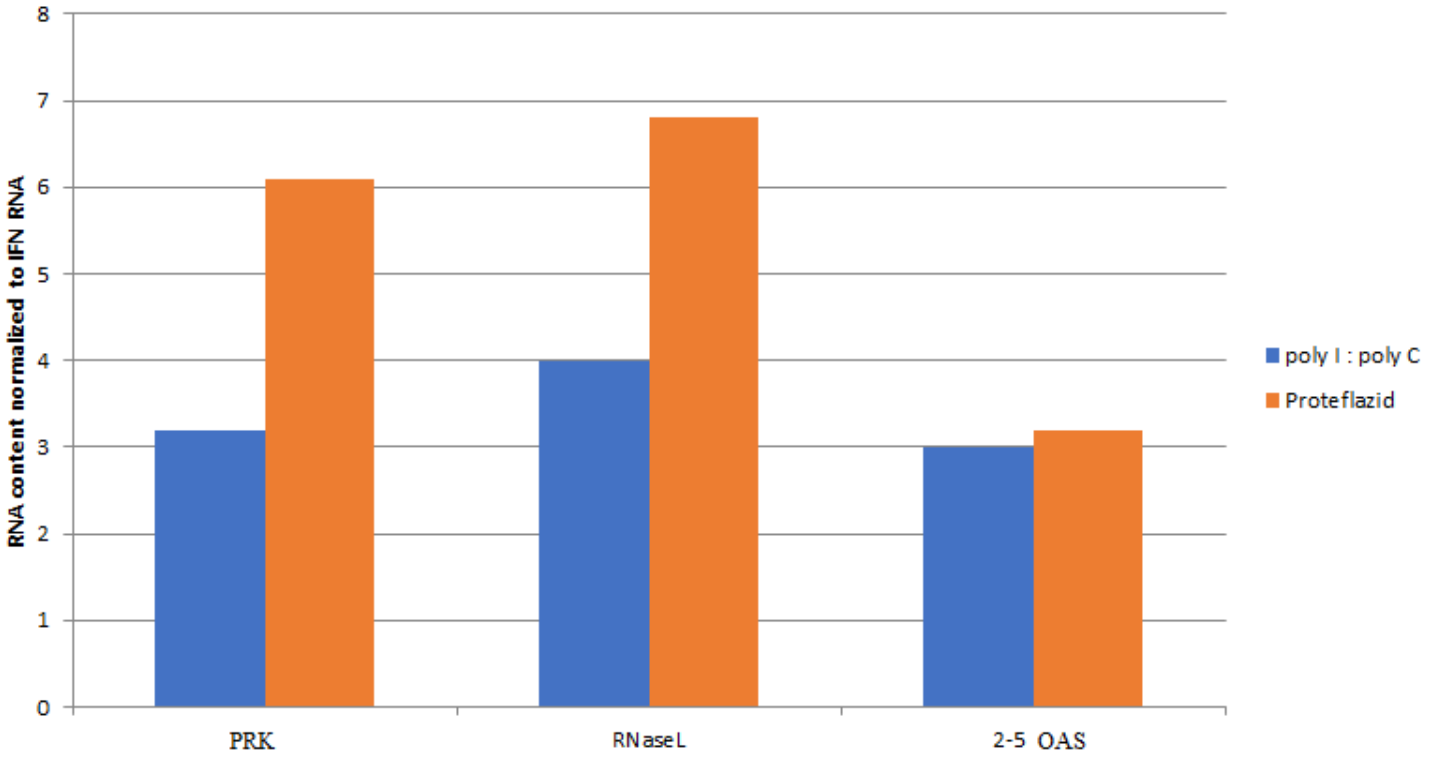
PROTEFLAZID – INTERFERON INDUCTION *IN VIVO*



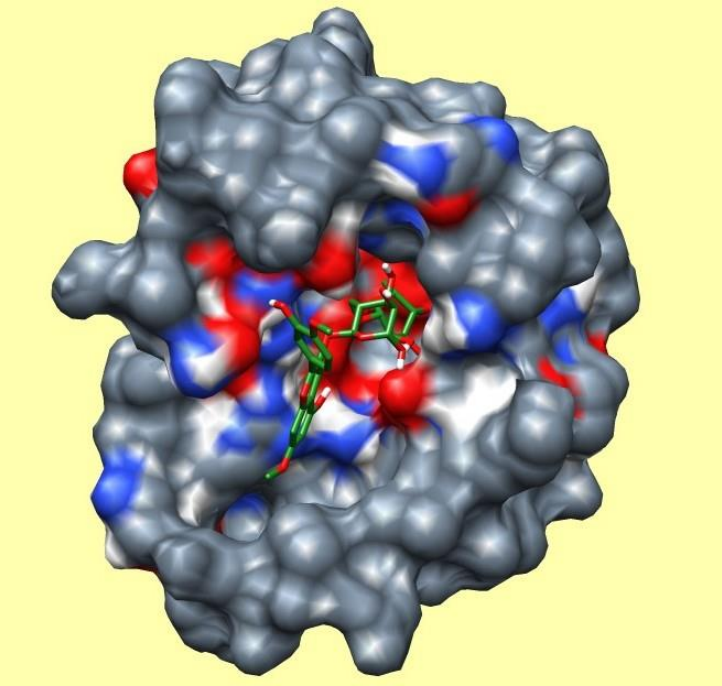
Interferon induction by Proteflazid in human leukocytes. The leukocytes were incubated with different concentrations of Proteflazid for 24 h at 37°C . Then in supernatants of human leukocytes the titer of interferon was estimated by the inhibition of VSV reproduction in A549 cells. Interferon titer was defined as the reciprocal of the dilution resulting in the inhibition of cytopathic effect in 50% of wells.

EFFECT OF PROTEFLAZID ON EXPRESSION OF ENZYMES OF INTERFERON SYSTEM

Levels of mRNA of protein kinase, RNase L and 2-5 oligoadenylate synthetase induced by Proteflazid in rats



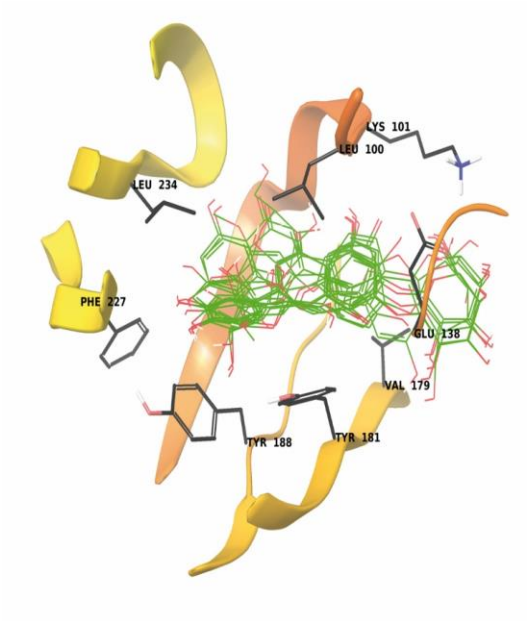
Binding modes of active flavonoids substances with neuraminidase of influenza virus based on docking analysis (QXP/Flo+,Chimera™) - computed model of interaction with neuraminidase active site



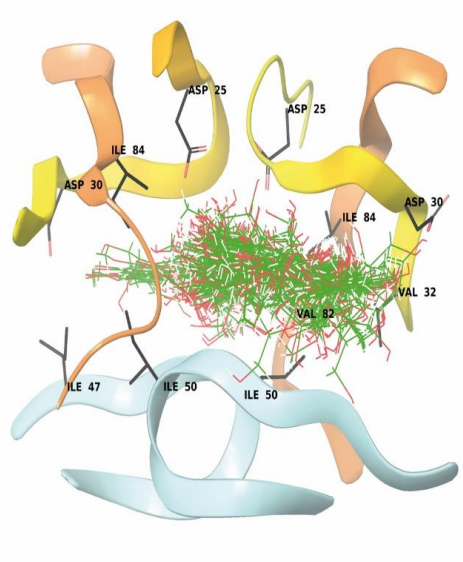
Neuraminidase as one of the molecular targets of active flavonoids substances (Proteflazid)

Possible mechanism – competitive inhibition wherein flavonoids compete for neuraminidase substrate – sialic acid residues

BINDING MODES OF FREE AGLYCONS OF FLAVONOID SUBSTANCES AND THEIR GLYCOSIDES WITH HIV PROTEASE BASED ON DOCKING ANALYSIS

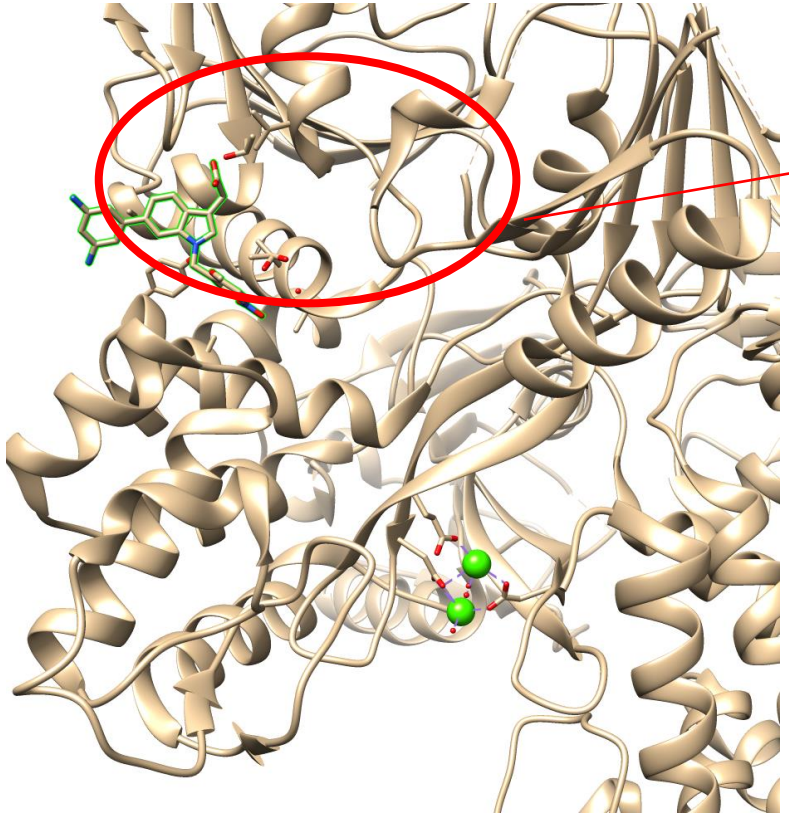


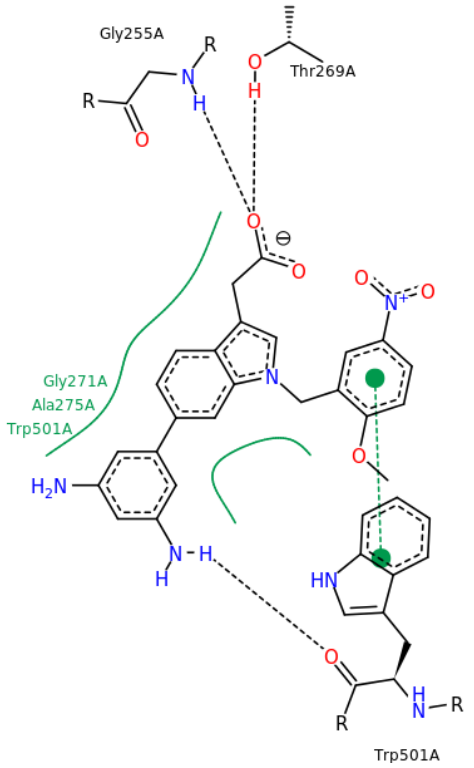
Binding modes of free aglycons and their glycosides with HIV reverse transcriptase based on docking analysis. Superposition of glycoside derivatives demonstrating the strongest binding with HIV reverse transcriptase. The HIV reverse transcriptase is depicted as the yellow and orange ribbons. The amino acid residues are represented as the rods and carbohydrate frame is colored in black. The free aglycons and their glycosides are depicted as the rods with their carbon atoms being displayed in green. Nevirapine is depicted as the rods with its carbon atoms being displayed in purple.



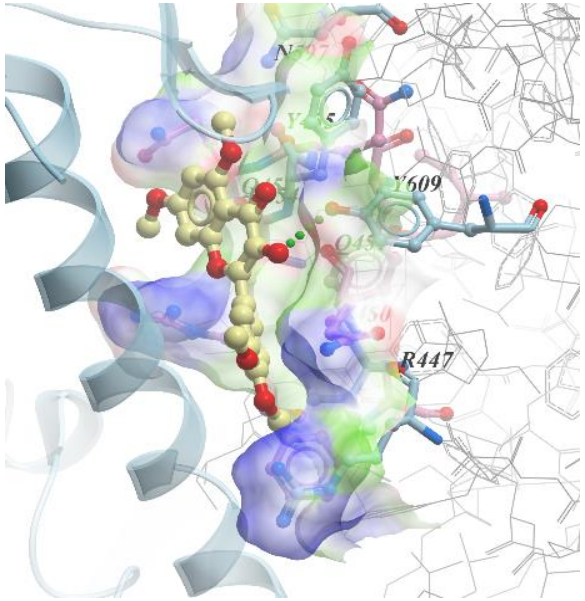
Binding modes of free aglycons and their glycosides with HIV protease based on docking analysis. Superposition of glycoside derivatives demonstrating the binding with HIV protease. The HIV protease is depicted as yellow ribbons (site of catalytic acids), orange ribbons (hydrophobic pouches) and blue ribbons (mobile flaps) with amino acid residues represented as the rods and carbohydrate frame colored in black. The free aglycons and their glycosides are depicted as the rods with their carbon atoms being displayed in green. Saquinavir is depicted as the rods with its carbon atoms being displayed in purple.

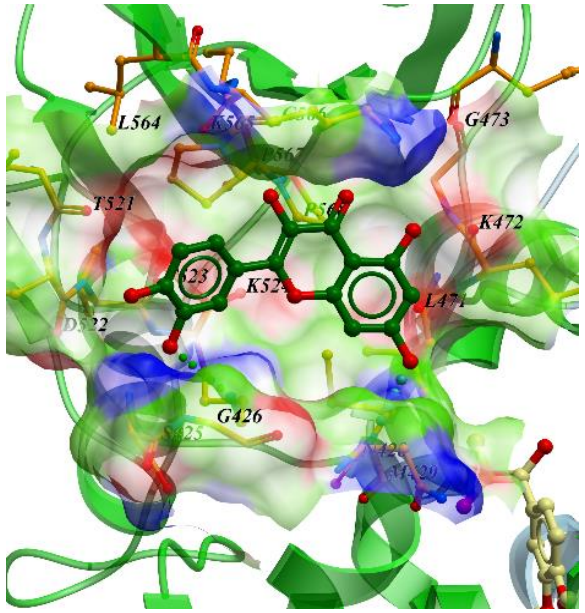
ACTIVE FLAVONOIDS SUBSTANCES (PROTEFLAZID) – DATA OF THE 4OKS COMPLEX OF HEPATITIS C VIRUS HELICASE



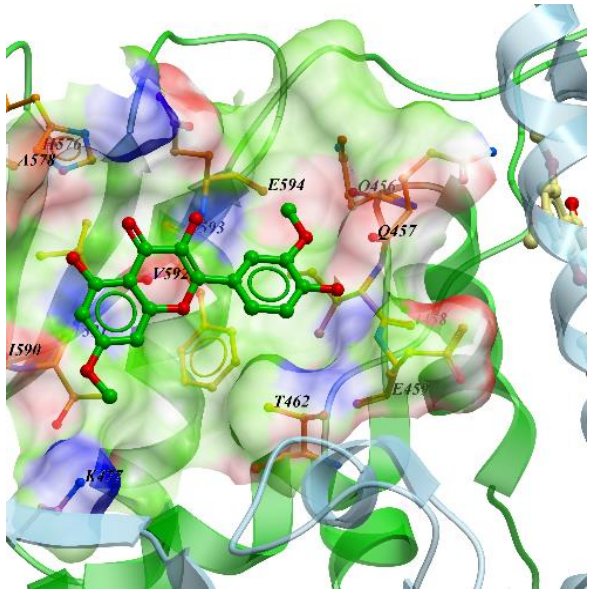


BINDING MODES OF ACTIVE FLAVONOIDS SUBSTANCES (PROTEFLAZID) WITH E1-E2 DIMER OF HPV BASED ON DOCKING ANALYSIS (INTERACTION WITH 3 POTENTIAL BINDING SITES IS SHOWN)

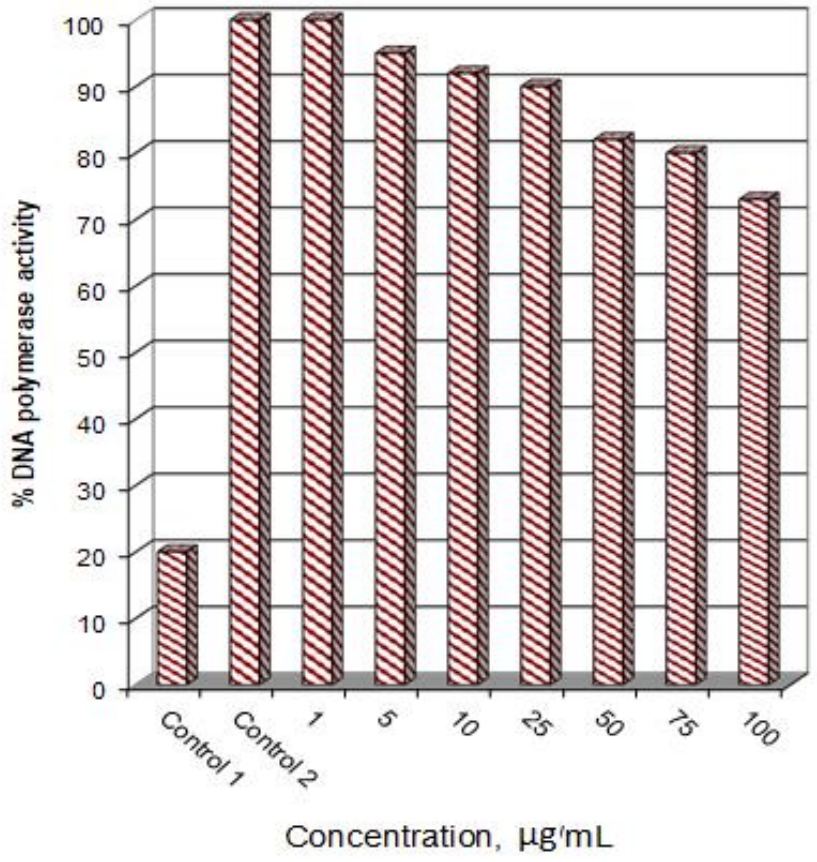


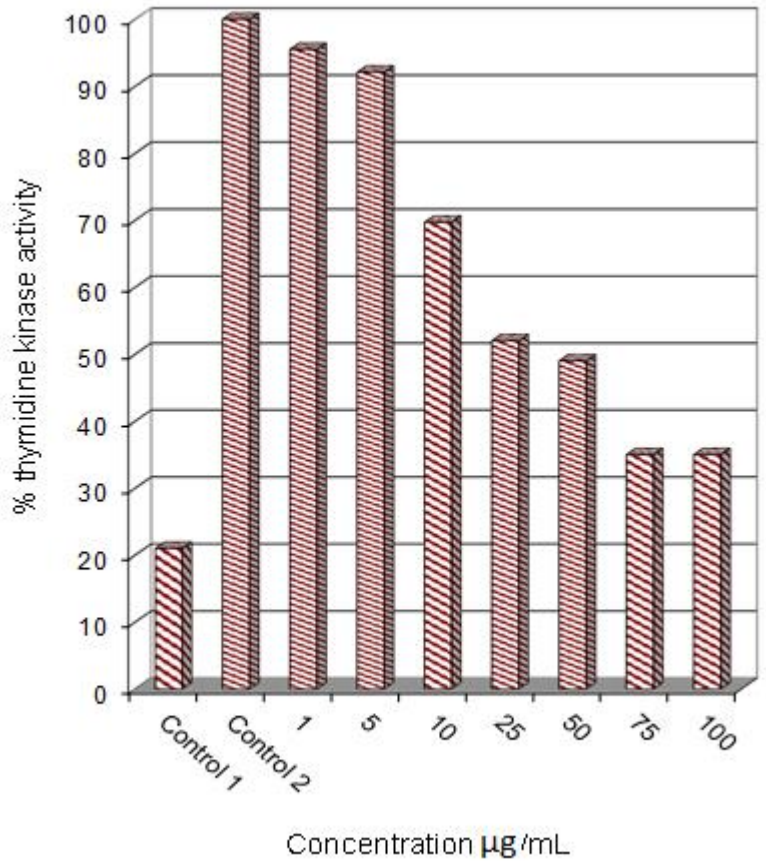


Helicase (E1) of HPV type 18



EFFECT OF PROTEFLAZID ON DNA POLYMERASE AND THYMIDINE KINASE ACTIVITY IN BRAINS OF MOUSE INFECTED WITH HSV





MODELING OF HUMAN PAPILLOMAVIRUS INFECTION *IN VITRO*: THE INFLUENCE OF PROTEFLAZID ON PAPILLOMAVIRUS REPRODUCTION

The problems of diagnosing and treating papilloma virus infection are in the spotlight taking into account the high prevalence of HPV infection and proved HPV oncogenicity. Infection with HPV is recognized as one of the major causes of infection-related cancer worldwide, as well as the causal factor in other diseases.

At present, over 100 HPV genotypes have been identified (Stoler 2000). HPVs that infect the genital tract are subdivided into low-risk and high-risk (HR) types. HR-HPVs (HPV-16, 18, 30, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 70, 85) are able to transform epithelial cells resulting in high-grade cervical lesions that can progress to cancer.

HPV DNA is detected more than in 99% samples of invasive cervical carcinoma. HPV HPV-16 и HPV-18 are the most common in cervical lesions and cause 60–80% of all invasive cervical cancers (Glifford et al. 2003). HPV-31, HPV-33 and HPV-45 are responsible for about 10% of cancer cases (Cohen et al. 2005).

Existing models for HPV reproduction in vitro:

Cell lines of cervical carcinoma: SiHa, CasKi–HPV-16, HeLa, $C_{4\text{-}1}$ HPV-18

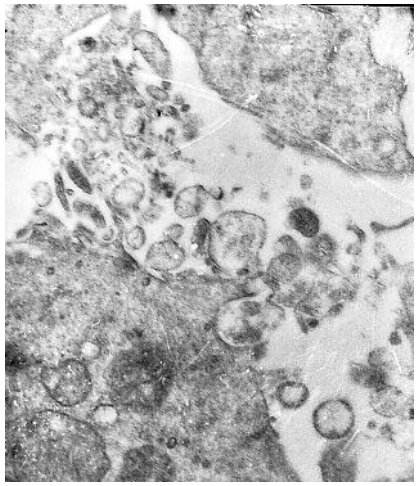
Co-culture of human condyloma explants with human fibroblast monolayer (Dollard et al., 1992) resulted in cell proliferation, HPV DNA integration and HPV expression

Transfection of HF-1 cells (human foreskin keratocytes) by HPV-16 DNA

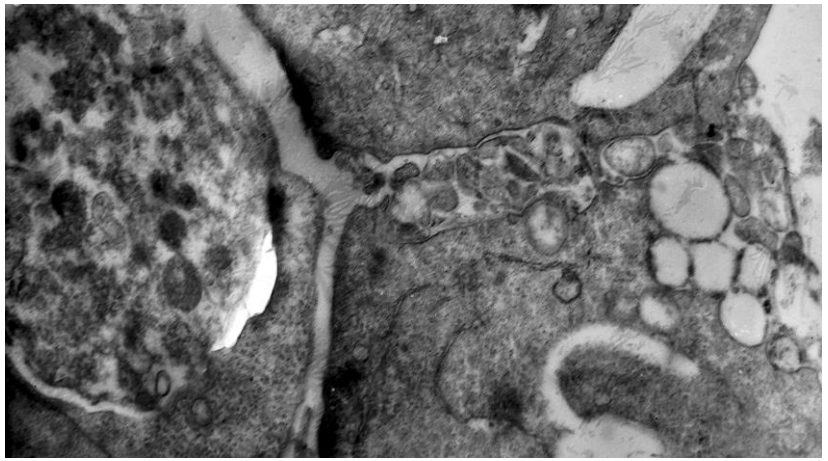
Except for vaccines, no HPV specific antiviral agents are available at present. Nevertheless, a vaccine can only prevent HPV infection, not cure an existing one. Therefore, the search for compounds capable of controlling HPV infection and inhibiting growth of HPV-infected cells is a relevant objective.

Polyphenols from natural and herbal extract are raising great interest as powerful and safe anticancer strategy for their broad range targeting capability and low side effects. In several studies both in vitro and in vivo polyphenols were demonstrated to inhibit the proliferation of HPV-immortalized and HPV-positive cancer cells, through induction of apoptosis, growth arrest, inhibition of DNA synthesis and modulation of signal transduction pathways. Among polyphenols, flavonoids are much less studied in this context, although they are shown to be effective in inhibiting reproduction of several other types of viruses and to inhibit selectively cancer cell growth

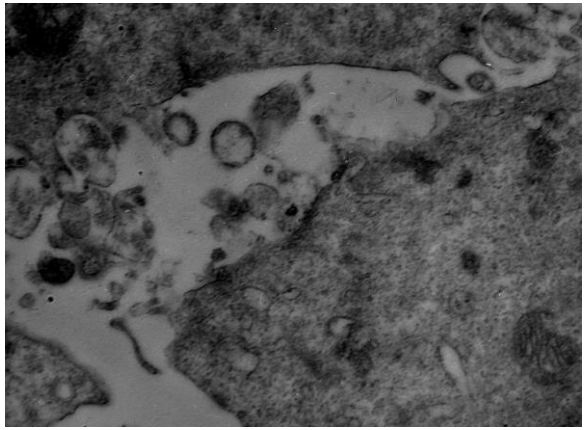
Models of papillomavirus infection in cells MT-4 and HeLa (electron microscopy)



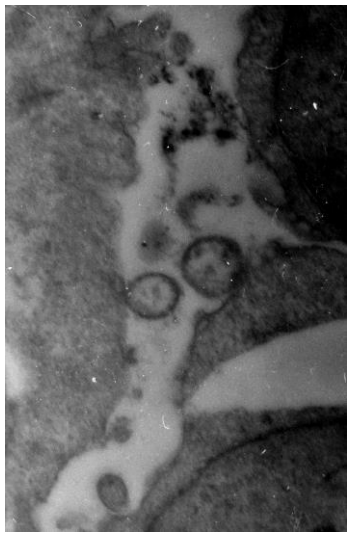
MT-4 cell+ HPV_59 type
Viral load - 5,25lg/10*5



MT-4 cell+ HPV_35 type
Viral load- 3,13lg/10*5

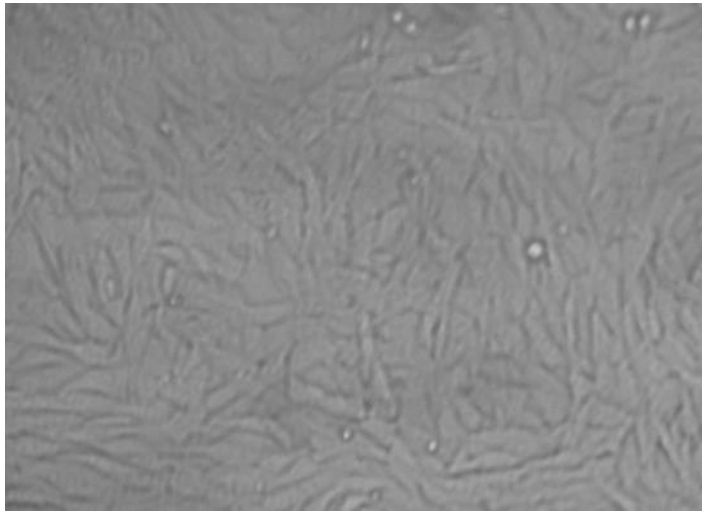


MT-4 cell+ HPV_16 type
Viral load - 4,15lg/10*5

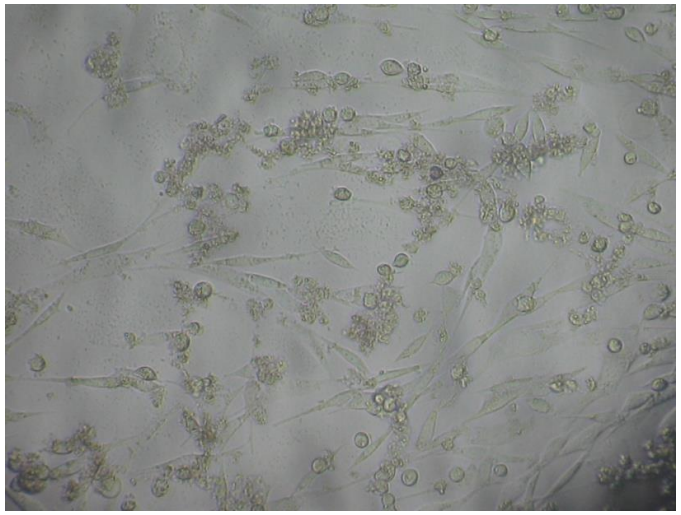


HeLa cells producing, HPV_18 type
Viral load – 3,45lg/10*5

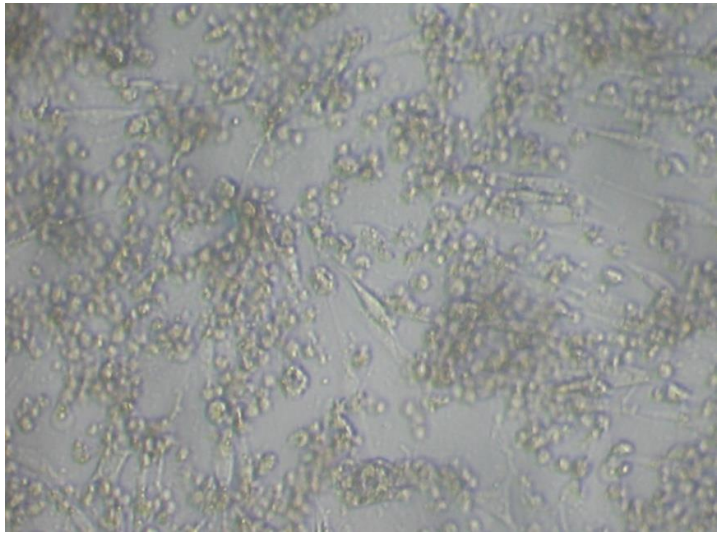
CYTOPATHOGENIC EFFECT OF HPV



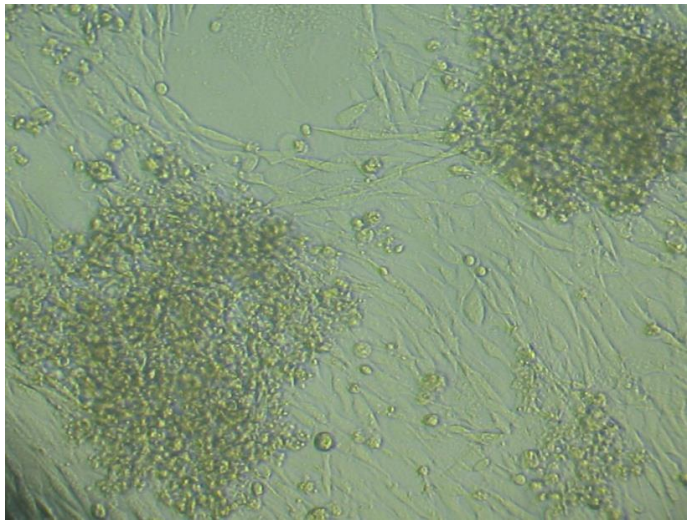
BHK control



BHK + HPV

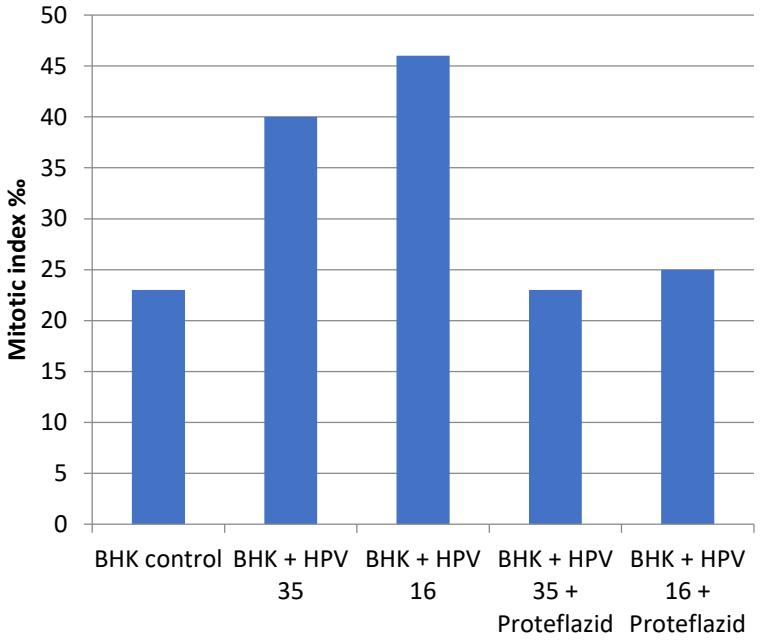


BHK +HPV

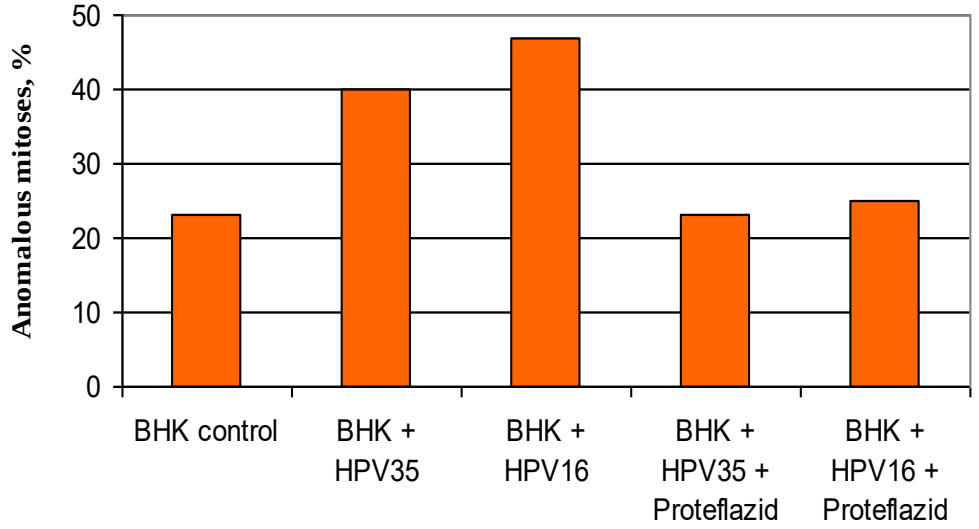


Focus of transformation of HPV

EFFECT OF PROTEFLAZID ON CYTOLOGICAL ACTIVITY OF PAPILLOMAVIRUS INFECTED CELLS



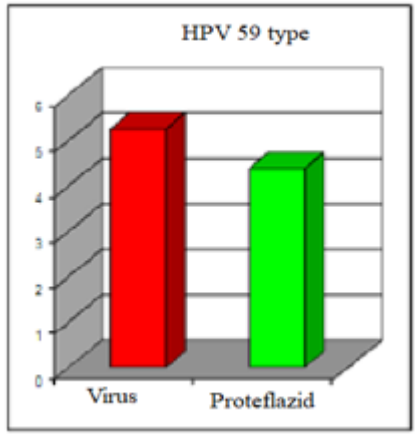
Effect of Proteflazid on mitotic index of HPV-infected BHK cells

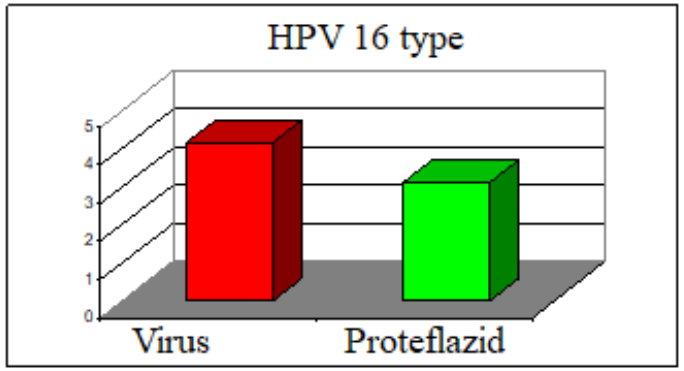


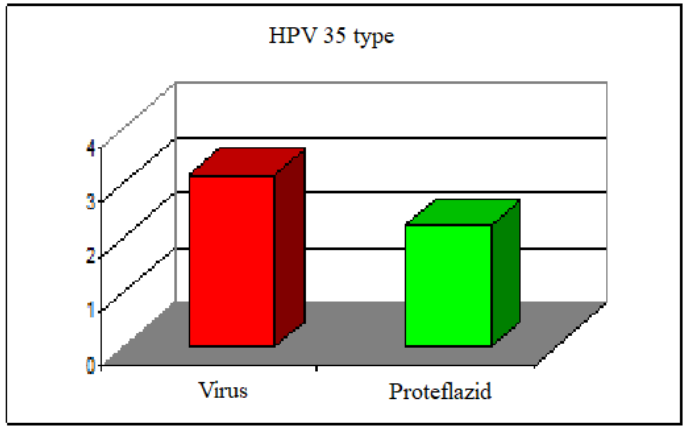
Effect of Proteflazid on anomalous mitoses rate in HPV-infected BHK cells

EFFECT OF PROTEFLAZID ON HPV LOAD IN CELL CULTURE

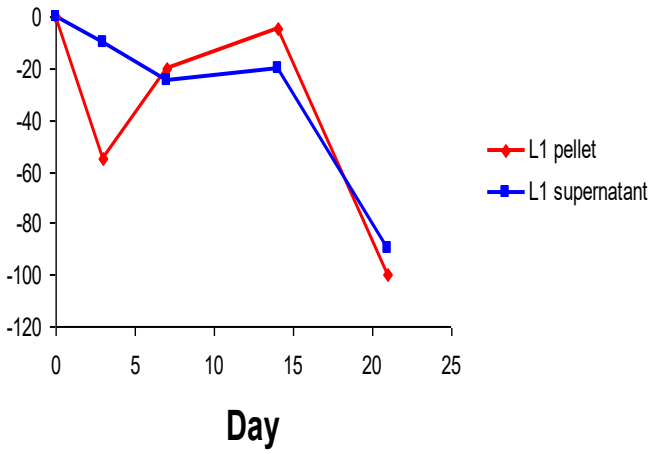
Proteflazid was administered 3 times (HPV load was tested in one month)	
	Viral load
HPV_59	**lg 5.25**
HPV_59 + Proteflazid	**lg 4.38**
HPV_35	**lg 3.13**
HPV_35 + Proteflazid	**lg 2.21**
HPV_16	**lg 4.15**
HPV_16 + Proteflazid	**lg 3.10**

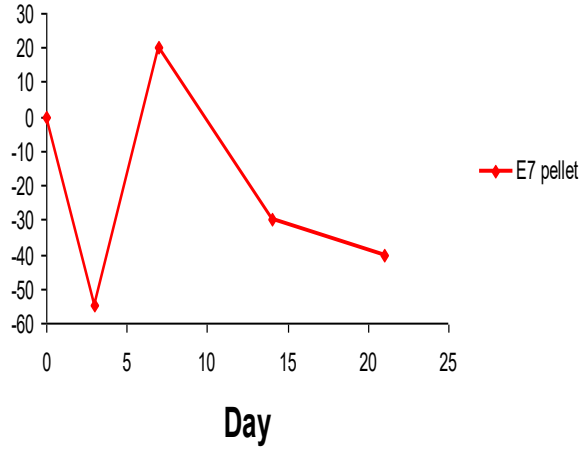


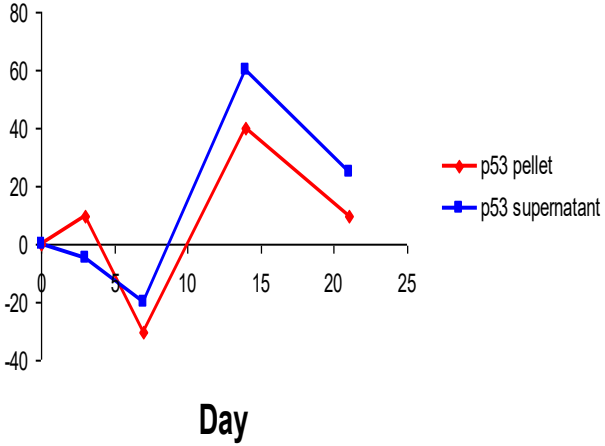


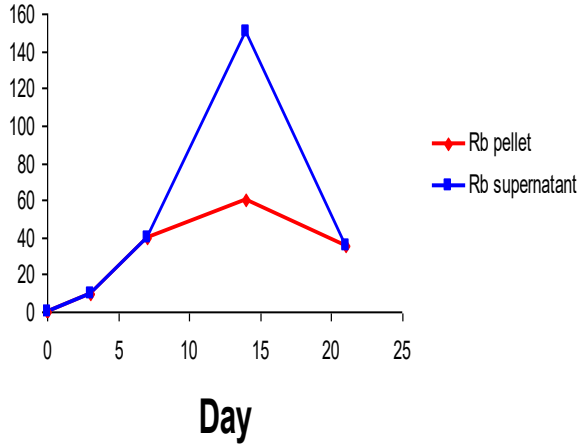


Effect of Proteflazid on expression of HPV proteins and cell proteins – onco-suppressors in MT-4 cells transfected by DNA of HPV 16 (assayed by ELISA with monoclonal antibodies)





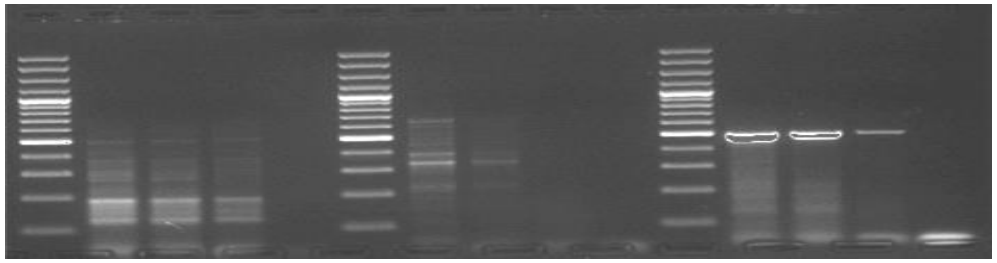




Effect of Proteflazid on expression of HPV proteins and cell proteins – oncosuppressors in MT-4 cells transfected by DNA of HPV 16 (assayed by ELISA with monoclonal antibodies)
The data are normalized to the expression in transfected cells not exposed to Proteflazid

Electrophoretic analysis of the products of HPV-16 virus with primers to the genes L,E1,E6 of HPV-16 virus

According to the data obtained, it is shown that the active flavonoids substances and Proteflazid inhibit the expression of the E1 and E6 genes.



M – 100 bp Plus DNA Ladder (Thermo Fisher Scientific)
№1- HPV16, №2 HPV16+Proteflazid , №3 HPV16 +active flavonoids substances
K- control minus HPV

Primer	Sequence (5'–>3')	Tm (° C)	GC (%)	Fragment size (bp)	Gene
dL1-F1	TTGTAAGCACGGATGAATATGTTG	53,7	37,5	269	L
dL1-R2	ACCACGACCTACCTCAACACCT	54,0	54,5		
dE1-f1	CAGTGATACAGGTGAAGATTTGGT	51,8	41,7	458	E1
dE1-r1	TATTTGTAAGTGGTGTTTGGCAT	51,0	34,8		
dE6-1F	CACAGGAGCGACCCAGAAAGT	55,6	57,1	332	E6
dE6-2R	CTTTTCTTCAGGACACAGTGGCTT	55,7	45,8		

MEDICAL PROBLEM

- **Human papillomavirus** (especially **HPV16 & HPV18**) is the cause of almost all cases of cervical cancer.[1]
- **Cervical ca**ncer ranks as the **3rd** most frequent cancer among women in the World

- **570,000** new cervical cancer cases are diagnosed and **311,000** deaths from cervical cancer annually[1]

[1] here and hereinafter: ICO/IARC Information Centre on HPV and Cancer, 2018

Today, there is no drug with specific antiviral effect on HPV that would eliminate HPV and thus prevent the development of cervical cancer in women who are diagnosed with HPV infection.

Project proposal “Innovative direct action anti-HPV agent for cervical cancer prevention” of SMC ECOPHARM LTD. was awarded by Seal of Excellence, issued by European Commission under Horizon 2020 Programme in February 2019

SEAL OF EXCELLENCE

European Commission

Certificate delivered by the European Commission, as the institution managing Horizon 2020, the EU Framework Programme for Research and Innovation 2014-2020

The project proposal **859790, UniVirTTM**

Innovative direct action anti-HPV agent for cervical cancer prevention

Submitted under the Horizon 2020's **SME instrument phase 2**
call **H2020-EIC-SMEInst-2018-2020 (H2020-SMEInst-2018-2020-2)** of **9 January 2019**
in the area of **EIC-SMEInst-2018-2020**

SME instrument
by
Scientific & Manufacturing Company Ecopharm Ltd.
9V Stepana Bandery Str.
04073 Kyiv
Ukraine

following evaluation by an international panel of independent experts

WAS SCORED AS A HIGH-QUALITY PROJECT PROPOSAL IN A HIGHLY COMPETITIVE EVALUATION PROCESS*

This proposal is recommended for funding by other sources since Horizon 2020 resources available for this specific Call were already allocated following a competitive ranking.

* This means passing all stringent Horizon 2020 assessment thresholds for the 3 award criteria (excellence, impact, quality and efficiency of implementation) required to receive funding from the EU budget Horizon 2020.

Corina Cretu, Commissioner for Regional Policy

Carlos Moedas Commissioner for Research Science and Innovation

Brussels, 18/02/2019

Project proposal «Innovative natural direct action antiviral agent for ARI treatment» of S&MCo. “ECOPHARM” LTD was awarded by Seal of Excellence, issued by European Commission under Horizon 2020 Programme in October 2019

SEAL OF EXCELLENCE

European Commission

Certificate delivered by the European Commission, as the institution managing Horizon 2020, the EU Framework Programme for Research and Innovation 2014-2020

The project proposal **890043, ARItreat**

Innovative natural direct action antiviral agent for ARI treatment

Submitted under the Horizon 2020's **SME instrument phase 1**
call **H2020-EIC-SMEInst-2018-2020 (H2020-SMEInst-2018-2020-1)** of **5 September 2019**
in the area of **EIC-SMEInst-2018-2020**

SME instrument
by
Scientific & Manufacturing Company Ecopharm Ltd.
9V Stepana Bandery Str.
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Corina Cretu, Commissioner for Regional Policy

Carlos Moedas Commissioner for Research Science and Innovation

Brussels, 09/10/2019

“Freedom from virus granted by science and nature”

✓ Study of the clinical efficacy of drugs based on the active substance Proteflazid

279 clinical studies, publications and preclinical studies confirmed the efficacy of active substance Proteflazid and the drugs on its basis, more than 20 000 patients participated, from them 4100 children and 1300 pregnant women

https://ecopharm.ua/en/science/papillomaviral-infection/systematic-reviews-and-meta-analysis-of-clinical-trials-4
https://ecopharm.ua/en/science/herpes-viral-infection/systematic-reviews-and-meta-analysis-of-clinical-trials
https://ecopharm.ua/en/science/influenza-and-other-acute-respiratory-viral-infections/systematic-reviews-and-meta-analysis-of-clinical-trials-3

✓ High safety profile – well tolerated, has no toxic, mutagenic, teratogenic, fetal-toxic effects.

✓ Form of release : drops, suppositories, syrup, capsules (Phase II)

"Scientific &Manufacturing Company “Ecopharm" LTD

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Please, write or call us , we will always be glad to meet you and we will send you our response!