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## **INTEGRATIVE THERAPEUTIC METHOD FOR SARS-CoV-2 INFECTION COMBINING FLAVONOID-BASED TRADITIONAL MEDICINE AND SILVER NANOPARTICLES**

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**Abstract:** There are evidences that flavonoids bind to and affect neutrophils, eosinophils and macrophages. In SARS-CoV-2, the cellular immune response, especially in the first two to three weeks after infection, is expressed by the accumulation at the infection site of at most white blood cell eosinophils, neutrophils, and monocytes/macrophages.

There have been numerous *in vitro* and *in vivo* studies on the antiviral activity of silver and copper nanoparticles, including in coronavirus infection. The our theoretical idea is that the silver nanoparticles or silver cations are transported by chelation from flavonoids to those white blood cells of the Cell-mediated immunity which are directly involved in the infection.

It was obtained and studied on Covid-19 infection a therapeutic mixture made by a controlled combining of wild basil (*Clinopodium vulgare* L.) tincture (66%, v/v) and other extracts of herbs rich in flavonoid and chlorogenic acid derivatives with a colloidal solution of silver nanoparticles and cations. It was found that the passing of the symptoms typical for the infection is for 3 to 10 days after the start of the treatment course. The highest percentage of patients with passed symptoms was reported from day 4 to day 6 (incl.) after initiation of dosing if therapy was initiated from day 1 to day 4 of the first symptoms. Based on this study, a Utility model "Composition for the treatment of coronaviruses" was registered with the Patent Office of the Republic of Bulgaria.

**Keywords:** flavonoid chelate complexes, silver nanoparticles, silver cations, SARS-CoV-2, cell-mediated immunity.

**Резюме:** Съществуват доказателства, че някои флавоноиди се свързват и взаимодействат с неутрофили, еозинофили и макрофаги. При Covid-19 клетъчният имунен отговор, особено през първите две до три седмици след инфекцията, се изразява с натрупване на мястото на инфекцията най-много от белите кръвни клетки еозинофили, неутрофили и моноцити/ макрофаги.

Има многобройни *in vitro* и *in vivo* проучвания за антивирусната активност на сребърни и медни наночастици, включително при коронавирусна инфекция. Нашата теоретична идея е, че сребърни наночастици или сребърни катиони се транспортират чрез хелатиране от съединения на флавоноидния ред до онези бели кръвни клетки от клетъчния имунитет, които са пряко участващи в инфекцията.

Беше получена и изследвана при инфекция с SARS-CoV-2 терапевтична смес, направена при контролирано комбиниране на тинктура (66%, v/v) от котешка стъпка (*Clinopodium vulgare* L.) и други екстракти от билки, богати на флавоноиди и производни на хлорогенна киселина, с колоиден разтвор на сребърни наночастици и катиони. Установено е, че преминаването на характерните за инфекцията симптоми е от 3 до 10 дни след началото на курса на лечение. Най-високият процент пациенти с преминали симптоми е отчетен от ден 4 до ден 6 (вкл.) след започване на приема, ако терапията е започнала от първи до четвърти ден след първите симптоми. Въз основа на това проучване в Патентното ведомство на Република България е регистриран полезен модел „Състав за терапия на коронавируси“.

### Introduction

Following the SARS-CoV pandemic and the efficacy of plant extracts containing large amounts of flavonoids, phenolic acids and other phenols, interest in their potential therapeutic use is focused on the SARS-CoV-2 virus (Russo, Maria *et al.*, 2020). Given the impact of the COVID-19 outbreak in China, it was expected that Traditional Chinese Medicine (TCM) could play a major role in the treatment of SARS-CoV-2 infection or at least in alleviating its symptoms. In this regard, by February 22, 2020, along with standard drug treatment, another treatment through TCM against COVID-19 was carried out in China in 60,107 patients. It was found that 92% of those infected with TCM reduced the recovery period by about 2 days, depending on the degree of development of the disease. Patients with mild clinical manifestations were in most cases treated with TCM alone. Only 5% of patients showed worse clinical signs (Publ. Dep. of the People's R. of China, 2020).

The Qingfei Paidu Decoction (QPD) formula is one of the most applied in this national study involving several thousand doctors. As a result of its effective effect, QPD is established as a prescription by the National Health Commission of the Republic of China in the sixth edition of the “Guide to Diagnosis and Therapy in Covid-19”. This formula consists of 21 components – herbs and minerals. Its administration showed an effectiveness of 92% in patients at all stages of disease development, including people cured and discharged. And in cases where clinical symptoms have disappeared, people remain stable without deterioration or significant improvement (Yang *et al.*, 2020). The beneficial effects of QPD are evident after 6 days of treatment with results of chest computed tomography in which the tracheobronchial shadow is normal, and the inflammation is also suppressed, following the theory and practice of QPD application (Ren *et al.*, 2020). In an attempt to identify the main components of QPD and investigate its pharmacological mechanism against COVID-19 infection, Yang *et al.* (2020) applied an integrated multidisciplinary approach – *in silico* method, which includes a pharmacological network and a molecular network of chemical analysis data. The UPLC-MS team identified in QPD 129 compounds grouped into 14 groups, where flavonoids accounted for about 45% of all groups, 15% glycosides, 10% carboxylic acids, 5% saponins and less than 3% terpenoids.

The action of various representatives of the flavonoid subgroups to suppress the development of viral infection by SARS-CoV 2 may be manifested by various cellular and biochemical mechanisms. One of them is the suppression of enzymes in the coronavirus cycle of infection. One of the main enzymes providing functional (non-structural) components of the virus is thought to be PL<sup>pro</sup> in SARS-CoV 2 and 3CL<sup>pro</sup> in SARS-CoV (Dai *et al.*, 2020). Other significant non-structural proteins of SARS-CoV-2 for its reproducible cycle are RNA-dependent DNA polymerase (RdRp) and helicase (Li and Clercq, 2020). SARS-CoV 2 PL<sup>pro</sup> was found to be significantly inhibited by the flavonoids tomatine A-E (IC<sub>50</sub> = 5.0-14.4 μM) (Cho *et al.*, 2013), bavachin, neobavaisoflavone, isobavachalcone, 4'-O-methylbavachalcone, psoralidin, corylifol A (IC<sub>50</sub> = 4.2-38.4 μM) (Kim *et al.*, 2014), quercetin, galocatechin gallate, epigallocatechin gallate (IC<sub>50</sub> = 47-73 μM) (Nguyen *et al.*, 2012), papyriflavonol A (IC<sub>50</sub> = 3.7 μM) (Park *et al.*, 2017), Xanthoangelol E (IC<sub>50</sub> = 1.2 μM) (Park *et al.*, 2016) and other flavonoid representatives (Russo *et al.*, 2020). NTP-ase/helicase possesses dsDNA looping activity as well as ATPase activity, allowing the helicase to translocate with nucleic acids by hydrolyzing ATP. According to Lee *et al.* (2009) quercetin, at IC<sub>50</sub> = 8.1 μM, inhibits SARS-CoV NTP-ase/helicase-based double-stranded DNA looping. And myricitin and scutularin are effective inhibitors of ATP-nitrogenactivity of helicase at IC<sub>50</sub> values 2.71 and 0.86 μM, respectively (Yu *et al.*, 2012). Miricitin and several glycosides of quercetin, as well as other phenolic compounds, exhibit both ACE2 and ACEIn inhibitory action (Kumar *et al.*, 2013; Patten *et al.*, 2016; Joshi *et al.*, 2020; Khaerunnisa *et al.*, 2020; Antonio *et al.*, 2020)

So far, evidence of inhibition of different stages of the coronavirus cycle of reproduction has been provided. In addition, studies have been carried out on dozens of compounds by *computer* molecular scanning for their potential impact of certain cellular mechanisms in coronavirus infection. TMPRSS-2 is already known to be involved in inoculation and replication of the virus influenza, SARS-CoV, SARS-CoV-2. Through the *in silico* method, several authors have suggested that several flavonoids have the potential to bind to TMPRSS-2, with particular reference to silibin /flavonolignan from milk thistle (*Silybum marianum* L.)/ (Pandit and Latha, 2020) and isogemichalcone B /(e.g. in representatives of the genus breadfruit (*Artocarpus*) / (Rahman *et al.*, 2020).

### ***Theoretical prerequisites for the establishment of the therapeutic approach***

Many of the representatives of flavonoids have anti-inflammatory, antihypertensive, antiangiogenic, hepatoprotective, antioxidant, neuroprotective and other actions in humans and animals (Kumar and Pandey, 2013). Particularly effective in viral infections, incl. Influenza A, subtype (H1N1, H5N2, H7N3 and H9N2) (Pantev *et al.*), SARS-CoV (Jo *et al.*, 2020), SARS-CoV-2 (Cherrak *et al.*, 2020; Russo *et al.*, 2020). Respiratory Syncytial Virus, Herpes simplex virus type 1 and 2, etc.<sup>28-29-30</sup> are the subgroups flavonols, flavones, isoflavones, chalcones (Zakaryan *et al.*, 2017; Jo *et al.*, 2020; Zandi *et al.*, 2011).

It is appropriate to present at least some aspects concerning the pharmacokinetics of flavonoids when interacting with certain leukocytes and their involvement in the cellular immune response. In the small and large intestine, flavonoids enter in their glycosidic or aglyconic form. Aglycons passively cross the intestinal barrier, while the transfer of flavonoid glycosides takes place via sodium-glucose-counter transporter-1 (Murota and Terao, 2003). Aglyconic forms were administered orally in native form or converted to such forms by enzymes hydrolyzing and releasing the glycoside residue. These enzymes are excreted by enterocytes and microbial flora in the gut (Murota *et al.*, 2018). In enterocytes or hepatocytes, aglycons are metabolised into glucuronidated, sulphated and/or methylated flavonoids (Day *et al.*, 1998). Glucuronidated forms of flavonoids are the most common representatives of flavonoids in lymphatic flow and blood plasma (Murota *et al.*, 2013). The lymphatic components and the flavonoids merge into the bloodstream through the thoracic canal. Quercetin has been reported to accumulate in rats in their

lungs (Boer *et al.*, 2005). There is credible evidence that flavonoids bind to and affect neutrophils (Amirova *et al.*, 2019; Tordera *et al.*, 1994; Kaneko *et al.*, 2017), eosinophils (Weng *et al.*, 2008; Middleton, 1998) and macrophages (Mendes *et al.*, 2019).

In SARS-CoV 2 (Covid-19), the cellular immune response, especially in the first two to three weeks after infection, is expressed by the accumulation at the infection site of at most white blood cell eosinophils, neutrophils, and monocytes/macrophages (Qin *et al.*, 2020; Gómez-Rial *et al.*, 2020; Sambataro *et al.*, 2020; Baker and Rogge, 2020). Neutralisation of the virus is induced there, as well as inflammatory and other reactions. At the same time, a decrease in the amount of these types of leukocytes is reported in the blood plasma in most cases. (Qin C *et al.*, 2020; Gómez-Rial *et al.*, 2020). This predominant reduction in blood samples of some types of white blood cells occurs in the majority of cases, and there is often a reduction in the first days after infection of other leukocytes, e.g. basophils.

The mechanism of action of different flavonoids on white blood cells may be different. Metabolic processes (in terms of pharmacodynamics) proposed to reflect flavonoid-mediated immunomodulation of macrophages include a decrease in glycolytic activity observed for all flavonoids tested; increased antioxidant protection; anti-inflammatory reprogramming of the Krebs Cycle (mainly by quercetin); osmotic regulation (by naringin) and membrane modification (by naringenin) (Gómez-Rial *et al.*, 2020). It is also essential that several isoflavones bind to macrophage's estrogen receptors (Mendes *et al.*, 2019; Kaneko *et al.*, 2015) as well as B cells, CD4+ T cells, CD8+ T cells, NK cells, plasma dendritic cells, monocytes and monocyte-derived dendritic cells (Kovats, 2015). The estrogen receptors of macrophage are characteristic in both women and men (Bhatia *et al.*, 2014).

The chemical composition of the herb wild basil (*Clinopodium vulgare L.*) collected in the Rhodope Mountains and/or in the vicinity of the town of Sofia at the stage of butonization – beginning of flowering shows a high content of flavonoids and other phenol compounds (Nikolova, 2011; Dagnon *et al.*, 2018). Flavones, rosmarinic acid, flavonols (Dagnon *et al.*, 2018) and chlorogenic acid (Bardarov, 2016; Amirova, 2019) predominate.

There are also studies that chlorogenic acid derivatives: caffeic acid, rosemary acid, chicoric acid, etc. have antiviral properties against Herpes simplex virus (HSV), Influenza A and immunodeficiency virus (HIV) (Utsunomiya H *et al.*, 2014; Langland *et al.*, 2018). However, the results of studies reported that the antiviral activity of caffeic acid increased more than 100-fold by adding cations such as Fe<sup>3+</sup> or anions such as molybdate and phosphate (Langland *et al.*, 2018). Tests for cellular toxicity of coffee acid chelates have shown that they have low toxicity. Coffee acid in chelate form with Fe<sup>3+</sup> has been tested against eight strains of viruses, including from different families. Coffee acid chelates are mostly effective against HSV-1 and HSV-2, but also exhibit moderate activity against Vaccinia virus and VSV-Ebola pseudotyped virus.

Data on the high chelating ability of many subtypes of the flavonoids with metal ions, such as Cu<sup>2+</sup>, Fe<sup>2+</sup>, Fe<sup>3+</sup>, Zn<sup>2+</sup>, is presented in many sources (Malešev and Kuntic, 2017). These cations are themselves oxidizers. However, most of their chelate complexes with flavonoids have higher antioxidant activity than the same flavonoids that are not bound to cations (Bravo and Anaconda, 2001; Mishra *et al.*, 2005; Dolatabadi, 2011; Malešev and Kuntic, 2017).

There have been numerous *in vitro* and *in vivo* studies on the antiviral activity of silver (Galdiero *et al.*, 2011) and copper nanoparticles, including in coronavirus infection (Jagaran and Singh, 2021; Jeremiah *et al.*, 2020).

In addition to metal cations, however, there is also evidence that some flavonoids also form complexes with metal nanoparticles through their free  $\pi$ -electrons and a carbonyl group. Quercetin and centin have the strong chelating ability with copper nanoparticles (Din *et al.*, 2017). Quercetin can also bind, cover and stabilize already obtained silver nanochatics (Shujahadeen *et al.*, 2019). The same applies to flavonoid-rich flax seed extract (*Linum usitatissimum L.*) (Ajum and Abbasi, 2016; Hussain *et al.*, 2017). and coral vine extract (*Antigonon leptopus*) (Ajum and Abbasi, 2016).

The aim of this investigation is to obtain and study on Covid-19 infected people the therapeutic mixture obtained by a controlled combination of wild basil tincture and other extracts of herbs rich in flavonoids with a colloidal solution of silver nanoparticles and cations.

## Material and Methods

### *Silver nanoparticles and herb extracts*

The silver colloidal solutions as a mixture of NP and cations are obtained by electrolysis and are a product of the company The Colloidal Company, England. The aqueous colloidal solution (25 ppm) is a mixture of silver nanoparticles (NP) and silver cations in a ratio of about 50:50 and is labelled as "silver water". The intake is according to the daily dose specified by the manufacturer, which is 2 times 8 ml per day.

Wild basil (*Clinopodium vulgare* L.) and the herbs contained in the extract with a trade name AI10HERB are collected in the region of Berkovitsa town. The ratio of dry herbs to ethanol-aqueous extractant is 1:10. AI10HERB consists of ethanol-water extract (66%, v/v) from Elderberry (*Sambucus nigra*), White thistle (*Silybum marianum*), Hoary willowherb (*Epilobium parviflorum*), Common nettle (*Urtica dioica*), Medical marsh mallow root (*Althaea Officinalis*), Ginger root (*Zingiber officinale*), Broadleaf plantain (*Plantago major*), Curcuma longa (*Curcuma longa*), Mead wort root (*Filipendula ulmaria*).

### *Preparation of chelate complexes*

To 8 ml of silver water add 20-30 ml of distilled water heated to 60-70 degrees Celsius. Immediately afterwards, 35-39 drops of 66% tincture of Wild basil are added and after 15 minutes of stirring periodically 40-45 drops of tincture AI10HERB of the company Herbalkan Ltd., are added. The described doses are for one intake. Daily intake is two doses.

### *Treatment study*

The two-stage study was conducted: I experimental period (stage) – 20.10-20.12.2020; II experimental period (stage) was additionally conducted because of validation experience – 15.01-28.02.2021.

*Stage I:* Randomized 37 volunteers in the study group (cohort) and 23 in the control group were enrolled. The effect of the therapeutic method was reported by clinical symptoms characteristic of the virus. An RT-PCR test was performed in all 37 patients and a positive test result was found. Volunteers are between 14 and 65 years old. Except for one participant at 14, the rest are in the range 32-65 years – an average of 43 years. In all participants, the use of this method was started between the first and the fourth day of the first symptoms, with *two or more of the following: fatigue, muscle pain, fever, decreased sense of smell, cough, anaemic-like symptoms*. It is reported on which consecutive day of the beginning of the treatment all the symptoms have passed established at the beginning of the course.

Volunteers between 26 and 63 years were enrolled for the control group – 44.5 years on average. An RT-PCR test was performed in all 23 patients.

Participants from both the control group and the experienced treatment group sought medical examination at the first symptoms, which was predominantly from the Family practice doctors. All subjects were assigned outpatient treatment according to the following model, which was administered equally in the experimental and control groups: zinc glucuronate, selenium methionine, vit. C, vit. D3 and probiotics in the recommended daily doses specified by the manufacturer; also 3 pcs. (1 morning and 2 evenings) Aspirin Protect 100 mg in case the patient does not take other anticoagulants. The patients in the control group who had continued symptoms until day 9, on our recommendation, sought rehabilitation and some of them were hospitalized. Most of the patients in the two consecutive control groups received inpatient treatment according to a treatment protocol from that hospital. We cannot give a specific figure for the number of hospitalized patients. According to the model /protocol/ for therapy presented in this article,

applied in the trial group, only out of hospital treatment under periodic supervision by a doctor has been carried out. There were no fatalities in the experimental or control groups.

*Stage II:* Randomized 87 volunteers in the trial group and 25 in the control group were enrolled. An RT-PCR test was performed in all patients The experimental group had an age range of 21-73 years (average 47 years) and the control group – 19-68 years (average 43.5 years). Again, in the experimental and control groups, an identical treatment protocol is applied as for Stage I. It is reported on which consecutive day of the beginning of the treatment all the symptoms have passed established at the beginning.

The statistical processing – by student's t-test using SPSS statistics (Kim, 2015) with Ms Office Excel 2010.

### Results and Discussion

Passage of the symptoms of the participants in the trial group at experimental period I is for 3 to 9 days after the start of the treatment course. From the data in table 1, the highest percentage of patients with passing symptoms was reported to be from the 4-th to the 6-th day (incl.) after the start of dosing – 70.3% (26 people), and the total for the period from the 3-rd to the 6-th day was about 75% (28 people). On the 7-th and 8-th days without symptoms were 21.6% (8 people) and on the 9-th day – 2.7% (1 person). *For the 10 days of therapy, only a positive effect in terms of the passage of COVID-19 typical symptoms in subjects was reported.*

In the control group, the participants with passed symptoms, except for 2 patients, was between the 13-th and the 26-th from the start of therapy, assigned by the respective attending physician whom they chose to visit. The majority of the symptomatic control group were 17-24 days. A reliable difference of the experimental sleep control variant is reported at  $p \leq 0.001$ .

Passage of the symptom of the participants in the trial group of the II experimental period is for 4 to 10 days after the start of the treatment course. The data in table 1 reported that the highest percentage of patients with passing symptom was from the 4th to the 6th day (incl.) after the start of dosing – 60.9% (53 people ); for the 7th-8th day without symptoms were 33.3% (29 people) and for the 9th-10th day – 5 people. A reliable difference of the experimental sleep control variant is reported at  $p \leq 0.001$ .

**Table 1.** Time by number of days from the beginning of the therapeutic course to pass the symptoms characteristic of SARS-CoV-2

	I experimental period		II experimental period	
	Trial Group Participants with resolved symptoms, n=37	Control group Participants with resolved symptoms, n=23	Trial Group Participants with resolved symptoms, n=87	Control group Participants with passed symptoms, n=25
3 <sup>rd</sup> day	2 (5.4%)			
4 <sup>th</sup> - 6 <sup>th</sup> day	26 (70.3%)		53 (60.9%)	
7 <sup>th</sup> - 8 <sup>th</sup> day	8 (21.6%)		29 (33.3%)	
9 <sup>th</sup> - 10 <sup>th</sup> day	1 (2.7%)		5 (5.7%)	1 (4.0%)
11 -16 day		2 (8.7%)		4 (16.0%)
17 - 22 day		15 (65.2%)		13 (52.0%)
23 <sup>rd</sup> - 26 <sup>th</sup> day		6 (26.1%)		7 (28.0%)
Mean day in order from the start of the Symptom Pass, Mean ± SD	*** 5.9 ± 1.25	20.4 ± 3.50	*** 6.4 ± 1.44	19.1 ± 4.34

Legend:  $p \leq 0,05$  (\* \*);  $p \leq 0,01$  (\* \*);  $p \leq 0,001$  (\* \* \*)

Our idea is that the elements effectively acting against the virus silver or copper (whether in the form of nanoparticles or free ions) are transported by chelation from compounds of the flavonoid order to those white blood cells of the cellular immune response that are directly involved in the infection and are in direct interaction with the virus. In addition to the effect, non-metal bound flavonoids have been shown to limit the replication cycle of the virus, as well as anti-inflammatory and other related actions (see introduction). We found that the pH of the mixture tincture of feline step + colloidal water in the given proportion is slightly above 7 and it is suitable for the formation of stable chelate complexes. The rationale for establishing this treatment method for certain aspects of the pharmacokinetics of the administered agents are also presented in this material.

Passage of the symptoms of the subjects in the experimental group at experimental period I is for 3 to 9 days after the start of the treatment course. The highest percentage of patients with resolved symptoms is reported from the 4th to the 6th day (incl.) after the start of the intake – 70.3% and the total for the period from the 3rd to the 6th day is about 75%. In the II experimental period, the passage of the symptoms is for 4 to 10 days, with the highest percentage for the 4th to 6th day after the start of the intake – 60.9%. Our opinion is that the applied mixture according to the author's method gives convincingly good results for claiming that it is suitable for significant assistance in the treatment after Covid-19 infection. The opinion is based on the percentage of infected patients with passed symptoms for the reporting periods compared to a control group.

### Conclusions

The mixture obtained by a controlled combination of wild basil tincture and other herb extracts rich in flavonoids, with silver colloidal water, have been shown to improve the healing process in case of SARS-CoV-2 infection.

It was found that the passing of the symptoms typical for the infection is for 3 to 10 days after the start of the treatment course. The highest percentage of patients with passed symptoms was reported from day 4 to day 6 (incl.) after the start of treatment, if the therapy was initiated from day 1 to day 4 of the first symptoms.

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