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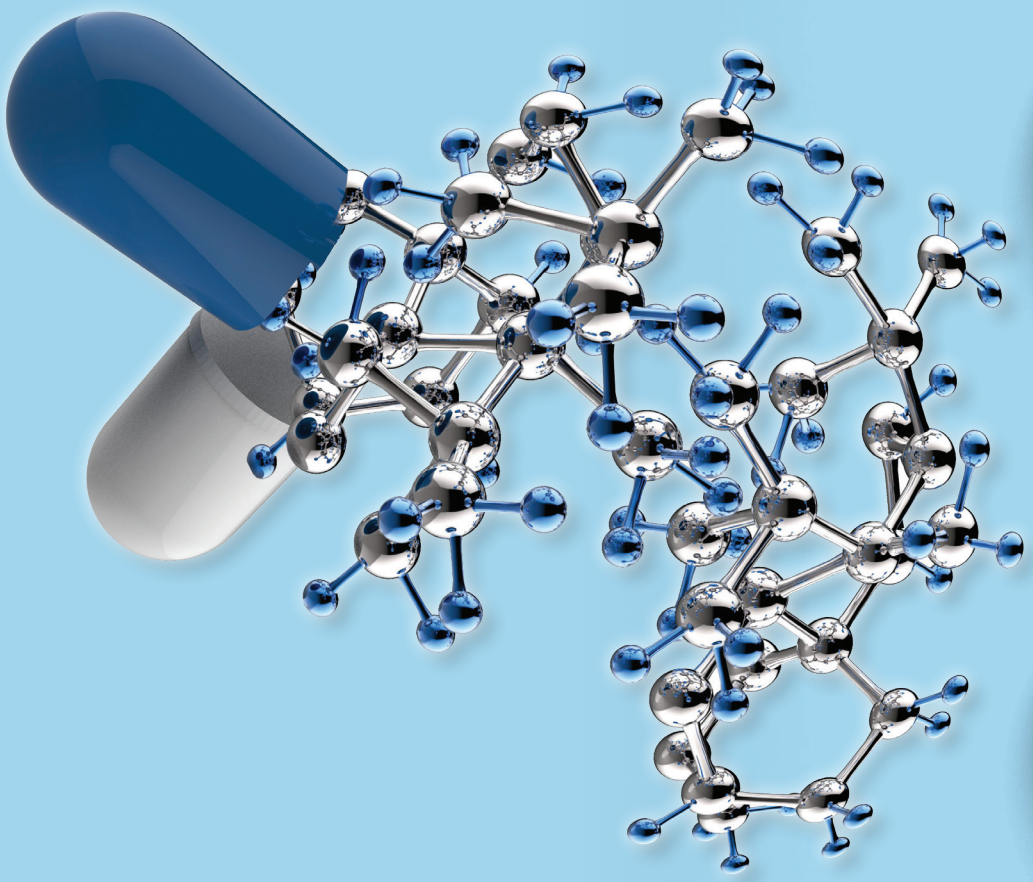
A QUARTERLY EDITION ON PHARMACEUTICAL SCIENCES' TOPICS



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ΠΕΡΙΕΧΟΜΕΝΑ / CONTENTS

Advances of Medicinal Chemistry against Kinetoplastid Protozoa (Leishmania spp). Infections: Drug Design, Synthesis and Pharmacology
Dimitrios Pavlos, Thomas Mavromoustakos ***254-271***

An Overview of Common Chemotherapeutics Used in COVID-19 Illness from Pharmaceutical Chemistry Point of View
Girija S. Singh ***272-291***

Determination of the concentration of low molecular fraction of candida albicans proteins by elisa method at intramuscular introduction in candidiasis therapy
Mykola Rybalkin, Tatyana Diadiun, Yuliia Azarenko, Serhiy Stepanenko, Olha Kaliuzhnaia ***292-295***

Pharmacological justification of the active substances in the composition of medicated chewing gum against the background of experimental xerostomia in rats
Yuliia Maslii, Dmytro Lytkin, Olena Ruban, Liudmyla Garmanchuk ***296-303***

Development and Validation of a Titrimetric Method for Quantitative Determination of Free Organic Acids in Green Tea Leaves
Olexander Maslov, Sergey Kolesnik, Mykola Komisarenko, Alexander Altukhov, Kateryna Dynnyk, Tatiana Kostina ***304-311***

ΦΑΡΜΑΚΕΥΤΙΚΗ

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ΦΑΡΜΑΚΕΥΤΙΚΗ, 33, IV, 2021 | 254-271 ΑΡΘΡΟ ΕΠΙΣΚΟΠΗΣΗΣ

PHARMAKEFTIKI, 33, IV, 2021 | 254-271 REVIEW ARTICLE

Advances of Medicinal Chemistry against Kinetoplastid Protozoa (Leishmania spp). Infections: Drug Design, Synthesis and Pharmacology

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KEYWORDS:
Leishmania spp., Life cycle, Leishmaniasis, Drug Design, Structure Based Studies, Ligand Based Studies.

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ABSTRACT

In this review article are described the detrimental effects of the parasite *Leishmania spp.* which is responsible for the disease of *Leishmaniasis*. Extensive reference is made on the description of kinetoplastids, *leishmaniasis* and the clinical forms of *leishmaniasis* and are provided relevant data related to Epidemiology, Intermediate hosts, Reservoir hosts, parasite evolution and Life cycle of *Leishmania*. Then, information is given on the currently available treatments and drugs. In the main part of this review article are analyzed the perspectives and current aspirations in the field of drug design that focuses on Structure Based Studies, Ligand Based Studies. Finally, the review article ends up with the integration of applied computational and experimental strategies of *leishmaniasis* drugs.

1. Introduction

1.1 Parasite *Leishmania spp.*, Description of kinetoplastids, *leishmaniasis*, clinical forms of *leishmaniasis*.

The parasite *Leishmania spp.* belongs to the genus of trypanosomes that are responsible for the disease of *Leishmaniasis*. The systematic classification of the parasite *Leishmania* is presented in detail in **Table 1**.

The parasite *Leishmania spp.* appears in two morphological features: The first one is the extracellular promastigote form. In this form the parasite grows and multiplies in the intestine of the invertebrate, host and in parasite cultures. It is larger than the second one, the amastigote form *is shown in* ***Figure 1***.

Specifically, the promastigote form is elongated with a size of 9-12 μm x 2-3 μm³. A large spherical nucleus with a clear nucleus and peripheral chromatin appears in the center. Numerous ribosomes, Golgi apparatus, fat vesicles, granular and smooth endoplasmic reticulum and lysosomes are found in the cytoplasm. Near the base of the whip there is a modified type of mitochondrion containing a discoid kinetoplast, a characteristic structure of parasites belonging to the kinitoplastids.

The kinetoplast contains two types of circular DNA molecules, known as kDNAs, microcycles and macrocycles. Kinetoplastids is a group of flagellated protists belonging to the phylum Euglenozea which they include a variety of common free-living species, as well as a few important parasites, some of which infect humans.

Kinetoplastids are distinguished by the presence of a DNA-containing region, known as a "kinetoplast," in their single large mitochondrion. From the one hand the different kinetoplastid pathogens have a similar genomic organization and similar cellular structures and all undergo morphological changes during their life cycles and from the other hand they cause distinct human diseases and are transmitted by different insect vectors. The majority of studies that have investigated the host cell response to *Leishmania* have evaluated early macrophage responses initiated by promastigotes[4]. The promastigotes located in the intestine of the host (sandfly) begin to multiply and are transported to the anterior part of the intestine, where they become cyclic. The parasites at this stage are smaller in size, have a lower protein content and cause more infectious. This form occurs in the later stages of parasite development[5].

The second one is the intracellular amastigote form. In this form the parasite is found in the mononuclear / macrophages of the final host. The parasite at this stage has lost its whip and is small oval in shape with a nucleus in the center, with an eyelid in front of the nucleus and an axon. In the phagolysosomes of the mononuclear / macrophages of the final host, the post-cyclic parasites are transformed into amastigote form. The amastigote form is oval with a diameter of 2-6 μm x 1.5-2 μm and has an eccentric nucleus with a nucleus and a kietoplastid that occupy most of the cell. In addition the amastigote form contains an intracellular whip ***(Figure 1)***.

1.2 *Leishmaniasis*

It is a disease caused by protozoa of the genus *Leishmania*. The parasites multiply in various species of vertebrate animals, which act as reservoirs. The parasite reservoir plays an important role in the conservation and epidemiology of the parasite. The parasite is transmitted from the intermediate host that is the vertebrate animals to the final host which are the sandfly of the genus *Phlebotomus* and *Lutzomyia*. *Leishmaniasis* is widespread on all continents except Australia and Antarctica. It has been estimated 1.71 billion and 1.69 billion individuals live in areas that are suitable for *Cutaneous Leishmaniasis (CL)* and *Visceral Leishmaniasis (VL)* transmission, respectively[6] and therefore the probabilities of transmitting the disease are high.

1.3 Clinical forms of *Leishmaniasis*

There are about 20 species of *leishmaniasis* (some of them are mentioned in **Table 1**) that can infect humans and cause a range of diseases with a wide range of clinical manifestations. The clinical features vary depending on the parasite's characteristics and on the genetic aspects of the host that determine the effectiveness of the immune response. According to the clinical manifestations, *leishmaniasis* can be divided into *(1) cutaneous (localized and disseminated), (2) muco-cutaneous, and (3) visceral or kala-azar*[7]**.**

1.3.1 *Cutaneous Leishmaniasis (CL)*

CL is worldwide the most prevalent clinical form of leishmaniasis, and 90% of all CL cases occur in only seven countries: Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia, and Syria[8].

It usually produces ulcers on the exposed parts of the body, such as the face, arms and legs. There may be a large number of lesions – sometimes up to 200 – which can cause serious disability. When the ulcers heal, they invariably leave permanent scars, which are often the cause of serious social prejudice[10]. *Leishmania* species can now be identified relatively easily with modern molecular techniques enabling a more rational therapy choice[9].

1.3.2 *Mucocutaneous Leishmaniasis*

Mucocutaneous is an infection caused by a single called parasite transmitted by sand fly bites. *Leish-*

Table 1: Systematic classification of the parasite Leishmania [1]

Subking-dom	***Protozoa***								
Phylum	***Sarcomastigophora***								
Subphylum	***Mastigophora***								
Class	***Zoomastigophorea***								
Order	***Kinetoplastida***								
Suborder	***Trypanosomatina***								
Family	***Trypanosomatidae***								
Genus	Endotry-panum	Crith-idia	Lepto-monas	Leish-mania	Herpeto-monas	Blasto crithidia	Sauro-leishmania	Trypano-soma	Phyto-monas
Subgenus					***Leishmania***				
Complex	L. donovani	L. tropica	L. major		L. mexicana		L. aethiopica		Not pathogenic to man Old world: L. Arabica L. gerbilli
Species	L. archibaldi L. chagasi L. donovani L. infantum	L. killicki L. tropica	L. major		L. amazonensis L. gamhami L. Mexicana L. pifanoi		L. aethiopica		New world: L. aristidesi L. enrietii L. deanei L. hertigi

mania species of the *L. (Viannia) subgenus*, including mainly *L. braziliensis, L. guyanensis* and *L. panamensis,* give rise to *CL* but are also responsible for *MCL* in up to 5- 10% of cases. *MCL* is clearly distinguishable from other *cutaneous leishmaniases* by its chronic, latent and metastatic behavior[11]. In *mucocutaneous leishmaniasis*, the lesions can lead to partial or total destruction of mucous membranes of the nose, mouth and throat cavities and surrounding tissues[10].

1.3.3 *Visceral* or *kala-azar Leishmaniasis (VL)*

The disease is endemic in rural India, Nepal, Bangladesh, Sudan and Brazil[12].

VL is the most serious of the three types of disease. In this type the parasite is systematically attacking and spreads throughout the body, infecting the body's macrophage cells, which then carry the parasite to the spleen, lymph nodes, liver and bone marrow. The incubation period, although difficult to determine exactly, ranges from 2-6 months.

Principal method of diagnosis is by the demonstration of amastigotes, i.e. LD bodies, either in the bone marrow or splenic aspirate[13]. Typical symptoms of the disease are intermittent fever, hepatomegaly and splenomegaly, lymphadenopathy, night sweats, fatigue, weakness, anorexia, diarrhea, gradual weight loss, vomiting, cough, skin ulcers. The hematological findings are: leukopenia, thrombocytopenia, anemia and hyper-γ-globinemia.

If the disease is not treated, the fatality rate in developing countries can be as high as 100% within 2 years[10].

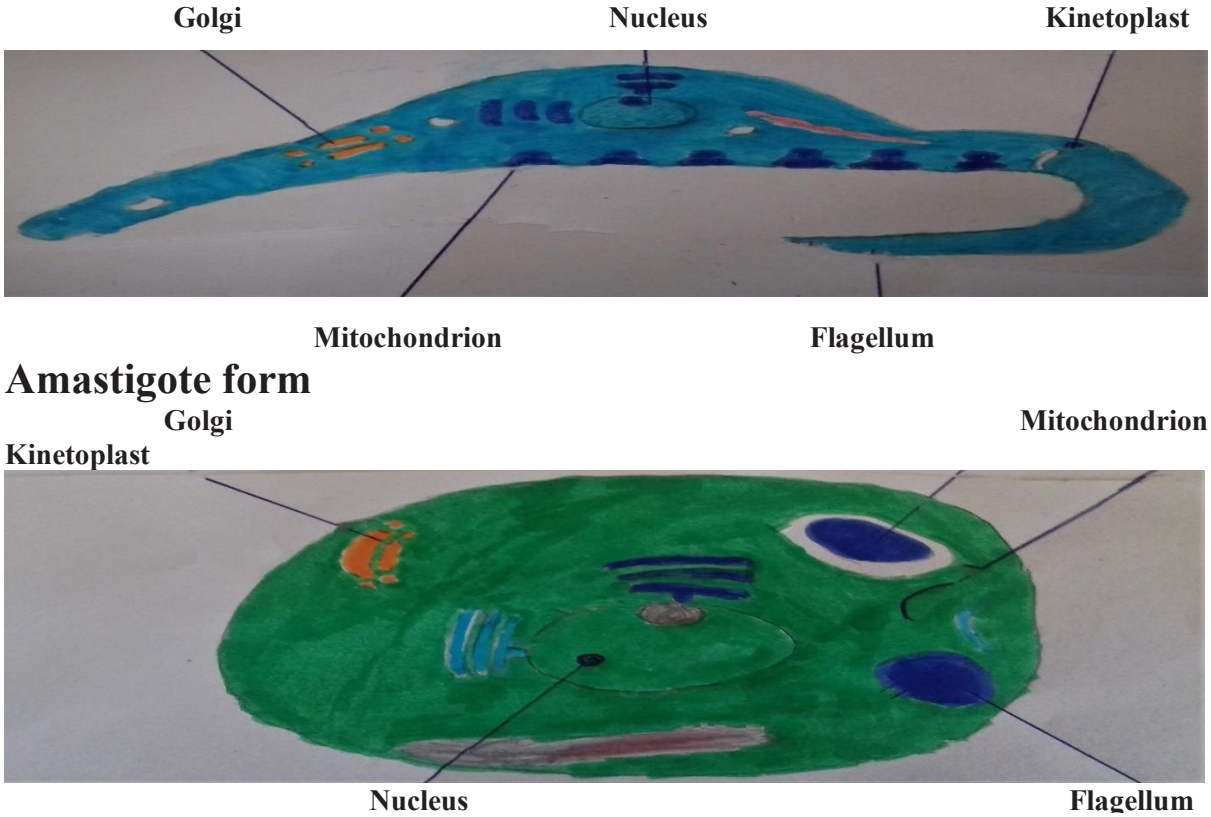


Figure 1. *Morphological forms of Leishmania sp. (A) promastigote form and (B) amastigote form. Arrows indicate key structures of the parasite, namely nucleus, kinetoplast, mitochondrion, Golgi and flagellum (adapted from Sankar P. D. et al.)* [2]

1.4 Epidemiology, Intermediate hosts, Reservoir hosts, parasite evolution, Life cycle of *Leishmania*.

Leishmaniasis epidemiological status is unevenly situated in different parts of the world[15].

The climatic conditions, the species of parasite that prevail in each area, the intermediate hosts, the final hosts as well as factors related to the above such as houses in the areas of reproduction / rest of sand fly species, type of house, immune status and host profession and infectious power of the strain are mainly the key factors that determine the observed temporal and spatial difference in the epidemiology of *leishmaniasis*[16, 17].

The epidemiological role of the various mammalian reservoir hosts is an important factor in controlling and eliminating *leishmaniasis*[19, 20]. This should be the target of epidemiological studies.

The transmission of the parasite is done with different species of sand flies that vary depending on the country where they live. There are over 600 species of sand flies divided into five genera: *Phlebotomus* and *Sergentomyia* in the Old World and *Lutzomyia, Brumptomyia* and *Warileya* in the New World [21, 22, 23]. In the Old World (Europe, Asia, Africa) the *leishmania* parasite is transmitted by the bite of infected female sand flies *Phlebotomus* and in the New World (America) by the bite of infected female sand flies *Lutzomyia*[18] **(Table 3)**. Each sand fly species

Table 2: Type of parasites and Geographical Distribution of Cutaneous Leishmaniasis		
Old World species		
Type of parasite	Clinical Form	Geographical Distribution
(L.) major	CL	Mediterranean basin, Middle East, the horn of Africa, and the Indian subcontinent.
(L.) infantum	CL	Mediterranean basin, the Middle East, the horn of Africa and the Indian subcontinent.
(L.) tropica	CL	Mediterranean basin, Middle East, the horn of Africa, and the Indian subcontinent.
New World species		
Type of parasite	Clinical Form	Geographical Distribution
(L.) amazonensis	CL	Middle and South America
(L.) chagasi	CL	Middle and South America
(L.) Mexicana	CL	Middle and South America
(L.) (V.) naiffi	CL	Middle and South America
(L.) (V.) braziliensis	CL	Middle and South America
(L.) (V.) guyanensis	CL	Middle and South America

typically transmits only one species of parasite and each parasite leads to a particular type of disease[24].

Sand flies *Phlebotomus* appear where there is large vegetation with moisture and a suitable temperature. They are small diptera insects 2-3 mm in size. Their body color is yellowish and after sucking blood they show a red spot. Adults emerge from the pupae after about one to two weeks. The whole cycle takes thirty to sixty days unless the larvae diapause, that may take four or five months[25].

Transmission usually takes place during the warmer months (April- October) and mainly between sunset and dawn[26]. It should be noted that the entry and installation of the parasite in the final host is greatly facilitated by sand flies saliva, which contains pharmacologically active substrates that generally inhibit the hemostatic mechanisms of the host and cause vasodilation and local immunosuppression and has been found to increase the infectivity of *Leishmania* [27].

At *zoonotic leishmaniasis* the reservoir hosts are wild animals, common animals or pets. At *anthropogenic leishmaniasis* the reservoir host is human. It should be mentioned that ecological and parasitological analysis are necessary to determine the role of animals as a reservoir in a given environment. The dog is the main reservoir host in *zoonotic visceral leishmaniasis*, caused by the parasite *L. infantum*. In some areas it may be considered that some wild animals (fox, jackal, rodents, wolves) can also act as reservoirs of the parasite[28].

Man is considered a random host and enters the above circle as follows: The construction of mud walls and earthen floors, the breeding of cattle and other animals near human dwellings, the human behavior of keeping pets in the house and its surroundings attract sand flies to the human settlement and therefore favor the transmission of the disease to humans[29].

Table 3: Sand fly species and geographical distribution[24]

Sand fly species	Geographical distribution
Phlebotomuspapatasi, Phlebotomusdubosqi, Phlebotomussalehi	Central and West Asia, North Africa, Sahel of Africa, Central and West Africa
Phlebotomussergenti	Central and West Asia, North Africa
Phlebotomuslongipes, Phlebotomuspedifer	Ethiopia, Kenya
Phlebotomusargentipes, Phlebotomusorientalis, Phlebotomus martini	Indian subcontinent, East Africa
Phlebotomusariasi, Phlebotomusperniciosus	Mediterranean basin, Central and West Asia
Lutzomyialongipalpis	Central and South America
Lutzomyiaolmecaolmeca	Central America
Lutzomyiaflaviscutellata	South America
Lutzomyiawellcomei, Lutzomyiacomplexus, Lutzomyiacarrerai	Central and South America
Lutzomyiaperuensis, Lutzomyiaverrucarum	Peru
Lutzomyiaumbratilis	South America
Lutzomyiatrapidoi	Central America

Human population movement also from endemic rural environments into urban areas is a major reason for the establishment of *leishmaniasis* in peri-urban and urban settlements[30, 31].

The biological life cycle of the parasite includes:

a) Development of the parasite in an intermediate host.

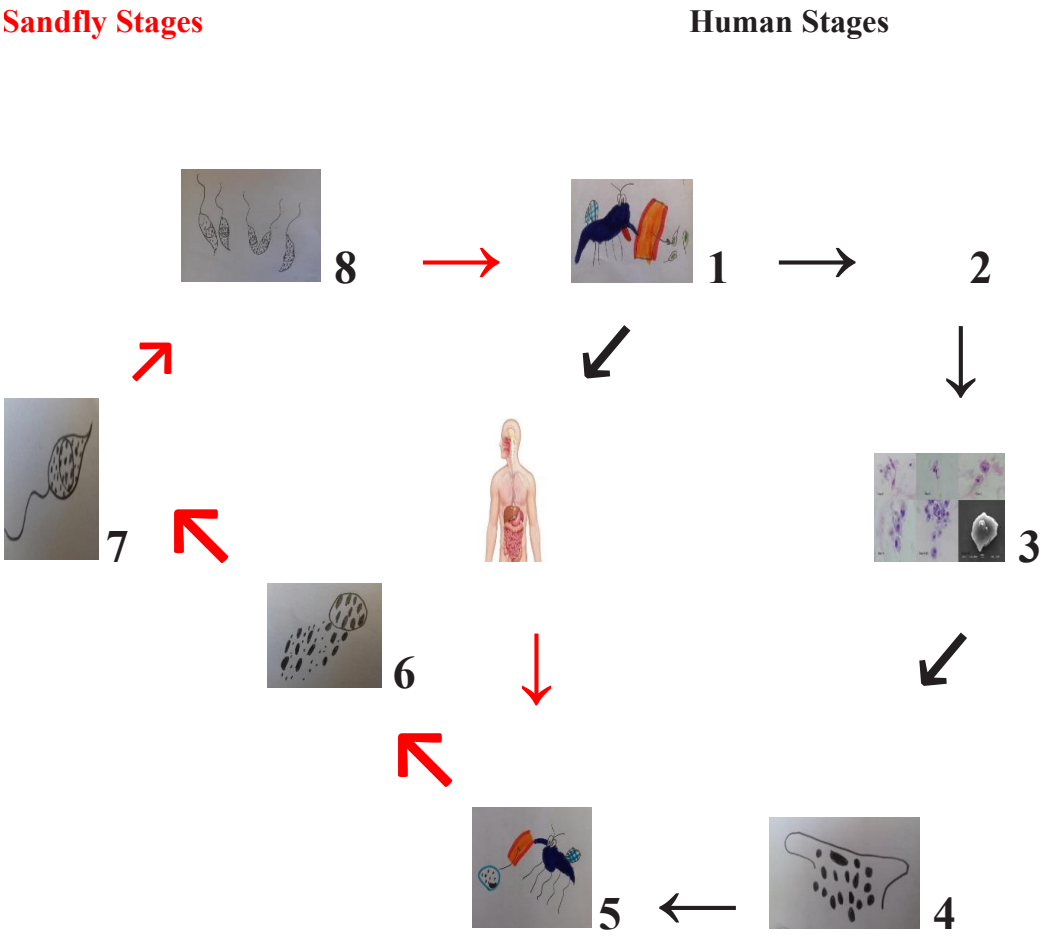
b) Development of the parasite in the final host such as carnivores, rodents, etc. as well as man. ***(figure 2)***

1.5 Currently available treatments, Drugs.

The currently available treatments for various forms of *leishmaniasis* have disadvantages mainly due to widespread toxicity, ineffectiveness, parenteral route of administration affecting compliance, high cost, emerging drug resistance, and lack of access in regional areas[32.]

The first- line drugs for the treatment of *leishmaniasis* are those based on ***pentavalent antimony compounds*** (sodium stibogluconate under the brand name "Pentostam" and meglumine antimoniate under the brand name "Glucantime"). These drugs have been widely used in *Cutaneous* as well as *Visceral leishmaniasis* for the last 60 years. Fluctuations in drug efficacy in *VL, CL* and *MCL* are one of the problems encountered in this type of treatment regimen[33].

In case of failure of the pentavalent antimony,



1. ***Sandfly takes a blood meal (injects promastigote stage into the skin)***
2. ***Promastigotes are phagocytized by macrophages***
3. ***Promastigotes transform into amastigotes inside macrophages*** [14]
4. ***Amastigotes multiply in cells (including macrophages) of various tissues***
5. ***Sandfly takes a blood meal (ingests macrophages infected with amastigotes)***
6. ***Ingestion of parasitized cell***
7. ***Amastigotes transform into promastigote stage in midgut***
8. ***Divide in midgut and migrate to proboscis***

Figure 2. *The life cycle of Leishmania species (adapted from Centers from Disease and Prevention)* [60]

pentamidine (pentacarinat), ***(figure 3)*** is used. Pentamidine is an antiprotozoal agent. It is an aromatic diamidine and is known to have activity against Pneumocystis carinii. The exact nature of its antiprotozoal action is unknown. In vitro studies with mammalian tissue and the protozoan *Crithidia oncopelti (parasites that exclusively parasitise arthropods, mainly insects)* show that pentamidine in-

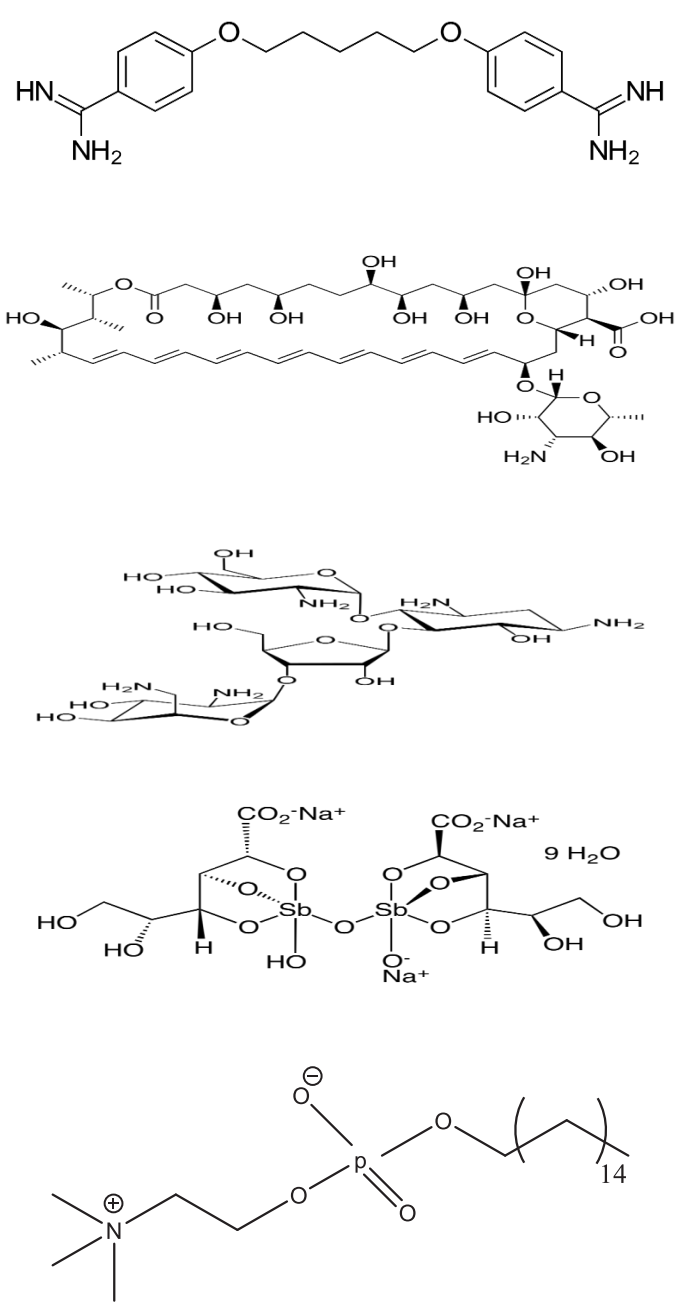


Figure 3*. The structural formulas of the following compounds are illustrated from top to bottom: Pentamidine, Amphotericin B, Paromomycin, Sodium Stibogluconate and Miltefosine*

terferes with metabolism and in particular inhibits the synthesis of DNA, RNA, phospholipids and proteins[34].

Amphotericin B (figure 3) is a macrocyclic antifungal of the polyene group isolated from *Streptomyces modocus*. The drug probably acts by binding to the sterols found in the cell membrane of the fungus, resulting in a change in the permeability of the

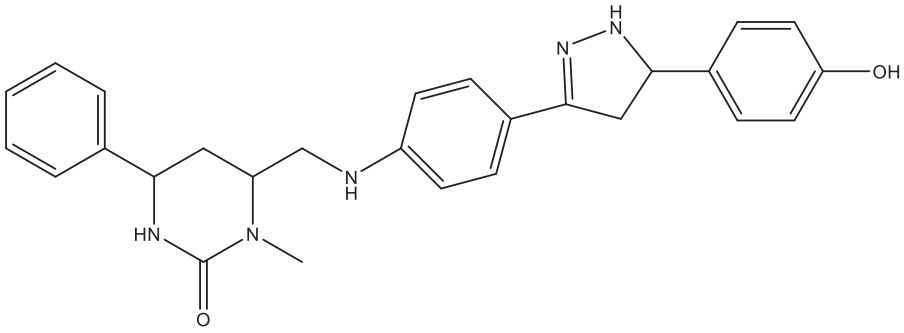


Figure 4. *The illustrated compound proved to be highly active against both L. major and L. donovani promastigotes, exhibiting a half-maximum inhibition concentration (IC50) of 948 nM and 3 μM, respectively* [48]

membrane that allows the leakage of various small molecules[34].

Liposomal amphotericin B is recommended by the World Health Organization (WHO) for the treatment of human leishmaniasis and is a new composition of the drug mentioned above, has reduced toxicity, longer half-life and high levels of activity in the treatment of *Visceral Leishmaniasis*[35].

Paromomycin (figure 3) is an antibiotic that fights bacteria. Paromomycin is used to treat certain *visceral* infections.

Paromomycin is also used to treat certain liver problems.

It has been observed that the best approach is to use a very potent drug with a short half-life in combination with a second drug having an extended half-life to clear the remaining parasites[36].

Thus the following combinations have been proposed and used:

-***Sodium stibogluconate (figure 3) and paromomycin*** for the treatment of *visceral leishmaniasis* under routine conditions in eastern Sudan has yielded much better results than monotherapy with Sodium stibogluconate [37].

-***Amphotericin B and Miltefosine (figure 3)*** in Combination for Treatment of *Post Kala-Azar Dermal Leishmaniasis*.

In the study of V. Ramesh et al. it was observed that patients treated with combination therapy demonstrated a rapid decline in parasite load and achieved 100% cure, with no reports of relapse[38].

-***L-AmB (Liposomal Amphotericin B), miltefosine and paromomycin***

In the study of S. Hendrickx et al., it was observed that implementation of paromomycin-miltefosine combination therapy indeed could represent a safe and affordable treatment option for *L. Infantum* as miltefosine appears to overrule the anticipated rapid development of paromomycin resistance[39].

2. Drug Design for *Leishmaniasis*

It is extremely important to mention that the discovery of drugs for *leishmaniasis*, similar to most early Neglected Tropical Diseases (NTDs)-focused research programs, relied on trial-and-error strategies that were based solely on phenotypic screenings.

In the mid-2000s, remarkable findings from genome projects began to open up opportunities, for the discovery of new drugs for *leishmaniasis* [40].

In recent years, collaborations between non-profit companies, pharmaceutical companies and academic institutions have been made available via open-access NTD-focused databases which have been essen-

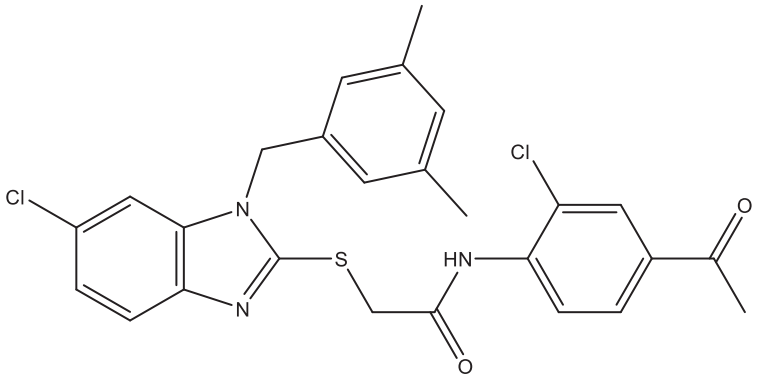


Figure 5. A new lead structure for further drug design of anti-*Leishmania* agents [49]

tial to the use of chemoinformatics in *leishmaniasis* research [41].

Some of the following extremely important databases have been created [42-45]:

1. WHO's TDR Targets Database (chemogenomics resource that is focused on NTDs and connects information from diverse protein and small-molecule libraries),
2. Sanger Institute's GeneDB (data of several Leishmania species),
3. LmSmdB (a database that simulates metabolic networks),
4. LeishMicrosatDB (a search engine for microsatellite sequences in *Leishmania genomes*)

As a consequence of the above more than 930 protein structures from *Leishmania spp.* are currently registered in the Protein Data Bank (PDB).

Thus, public-private partnerships (PPPs) have been developed to strengthen the research infrastructure of NTDs. Consequently, the combination of experimental evaluation and chemoinformatics studies have played a key role in the development of new *leishmaniasis* drugs that are in preclinical trials [46.]

Here are some promising macromolecular targets that have been investigated in *leishmaniasis* drug discovery: Topoisomerases, proteases (mainly cysteine-proteases), tubulin, proteins of the folate metabolic route, kinases, phosphodiesterases, enzymes that are involved in the trypanothione and purine salvage pathways. Have been also identified suitable Ligands for these targets, increasing the data level and favoring the conditions for the development of new drugs[47].

Thus the studies are divided into Structure- Based and Ligand Based rational design.

2.1 Structure- Based Studies

The extremely useful references presented below are the result of a coordinated scientific effort to find suitable structures to inhibit the action of the following enzymes which have been shown to increase the growth of the parasite *leishmania*: Pteridine reductase (PTR1), *Leishmania mexicana cysteine protease*, dehydrogenase-2 from *Plasmodium falciparum*, free *Leishmania donovani Topoisomerase 1* (LdTop1) the couple tryparedoxin/tryparedoxin peroxidase **(Table 5)**.

Pteridine reductase (PTR1) is an important enzyme responsible for Pteridine salvage in *leishmania* and other trypanosomatid protozoans. PTR1 contributes to antifolate resistance and is responsible for the failure of conventional therapies such as Methotrexate (MTX) against these protozoans [51].

Rashid et al. based on the fact that there were stud-

Table 5: Aggregate Data of Structure- Based Studies			
Research team	**Method**	**Target**	**Suitable ligands**
Rashid et al. [48]	Molecular docking studies	Pteridine reductase (PTR1)	3,4-Dihydropyrimidine
De Luca et al. [49]	Molecular docking studies	Leishmania mexicana cysteine protease	1,2-substituted-1H-benzo[d]imidazole derivatives
Stevanović S. et al. [50]	Molecular docking calculations	Dehydrogenase-2 from Plasmodium falciparum	6-methoxy-quinalidine
Mamidala R. et al. [53]	Pharmacophore modelling, X-ray crystallographic investigation and molecular docking studies	Free Leishmania donovani Topoisomerase 1 (LdTop1)	3,4-dihydroxyphenyl derivative
Brindisi et al. [54]	Comprehensive approach encompassing X-ray structure determination and a combination of High-Throughput Docking and preliminary hit optimization.	Tryparedoxin / Tryparedoxin peroxidase.	N,N-disubstituted 3-aminomethyl quinolone derivatives

ies reported 3,4-Dihydropyrimidine (DHPM) targeting PTR1 [52], focused on molecular docking studies to investigate PTR1 (from L. major) as a possible target for synthesized DHPM.

Crystal structure of PTR1 (PDB ID 1E7W, from *L. major*) with MTX as the co-crystallized ligand was selected for these studies Docking experiments were performed via Molecular Operating Environment (MOE) docking program.

They observed that compound illustrated in **figure 4** bound to PTR1 binding site showed that carbonyl oxygen of the cyclic urea act as a strong hydrogen bond acceptor and formed hydrogen bond with Lys198 of PTR1. Similarly, carbonyl oxygen of the C-5 acetyl group accepts hydrogen bond from Arg17. The observed negative fitness value of binding interaction (-8.9001 Kcal/mol) revealed that DHPM was not tightly fitted into the active site. The orientation of MTX and its hydrogen bonding and π-stacking interactions provide a framework for the novel scaffold design[48].

De Luca et al. research group discovered a new class of 1,2-substituted-1H-benzo[d]imidazole derivatives acting as non-covalent and selective inhibitors of the *Leishmania mexicana cysteine protease* CPB2.8ΔCTE, one of the most promising target within anti-*Leishmania* drug design[49].

The compound illustrated in **figure 5** showed:

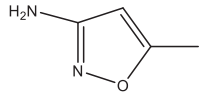
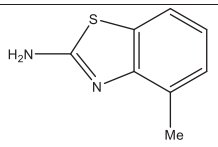
1. Affinity towards the enzyme in the submicromolar range (Ki = 0.69 μM)

2. Activity against the intracellular form of the parasite (amastigotes of *L. infantum*) in the micromolar range (IC_{50} = 6.8 μM).

3. Good oral availability and results in a non-mutagen and noncarcinogenic profiling.

Stevanović S. et al. developed and validated a three-dimensional homology model of the *L. infantum* NDH-2. Subsequently using reported 4-quinolone inhibitors of NDH-2 from Plasmodium falciparum (a unicellular protozoan parasite of humans, and the deadliest species of Plasmodium that causes malaria in humans), ligand-based pharmacophore

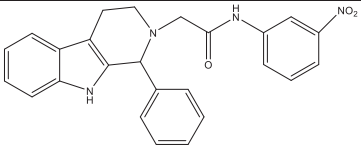
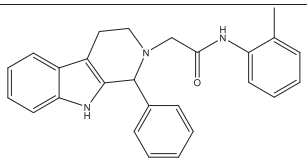
Table 6:α-aminophosphonates componets displayed potent inhibitory activity in the low (7.1– 9.75) mM range. These compounds exhibited a cytotoxic (against J774A-1 macrophages) profile similar to that of the standard drug amphotericin B [56]

Entry	R^1	R^2	R^3NH_2	Time (h)	Yield [b] %	IC_{50}[e] (μM)	CC_{50}[f] (μM)	SI[g] (CC_{50} / IC_{50})
1	4-(OH), 3-(OMe)-C_6H_3	H	$PhNH_2$	6	88	7.1(0.14)	5	0.70
2	4-(OH)-C_6H_4	H	$PhNH_2$	6	86	8(0.14)	6.5	0.81
3	4-(OMe)-C_6H_4	H		4	78[c]	8(2.9)	7.5	0.94
4	4-(OMe)-C_6H_4	H		5	76[c]	8.75(3)	8.5	0.97
5	4-(NO_2)-C_6H_4	H	$PhNH_2$	6	83	8.95(0.95)	5	0.56
6	2-(OH)-C_6H_4	H	$PhNH_2$	7	88	8.95(0.05)	5	0.56
7	3-(OH), 4-(OMe)-C_6H_3	H	$PhNH_2$	6	86	9.75(0.125)	31	3.18

models and virtual screening were coupled with subsequent molecular-docking calculations produced 54 hits with an inhibitory potential [50].

From the selected 23 compounds with the highest docking scores for their *in vitro* inhibitory activity towards recombinant NDH-2 from *Staphylococcus aureus* (a Gram-positive, round-shaped bacterium that is a member of the Firmicutes, and it is a usual member of the microbiota of the body, frequently found in the upper respiratory tract and on the skin) and their leishmanicidal activity for *L. infantum* axenic amastigotes and proamastigotes the most prevalent was 6-methoxy-quinalidine as an inhibitor of NDH-2 from *S. aureus* (Ki app = 8.9 ± 1.0 μM).

Table 7: Results of in-vitro anti-leishmanial activity of the most active tetrahydro-b-carboline derivatives [57]

Tetrahydro-b-carboline derivative	*CC_{50} (μm)*	IC_{50} (μm) Promastigotes	CC_{50}/IC_{50}	*IC_{50} (μm) Amastigotes*	*CC_{50}/IC_{50}*
	>200	>100	-	0.87 ± 0.09	>229.8
	>200	10.43 ± 0.44	>19.18	0.67 ± 0.05	>298.5

The IC_{50} results ranged between 200–300 nM against wild-type axenic amastigotes and IC50 results ranged between 30–50 nM against promastigotes for 6-methoxy-quinalidine favor the conditions for the development of a drug for the treatment of *leishmaniasis*.

In Mamidala R. et al. research paper [53] the most active 3,4-dihydroxyphenyl derivative, interacted with free *Leishmania donovani Topoisomerase 1* (LdTop1) as observed in the preincubation DNA relaxation inhibition experiment. The active compounds showed minimal toxicity when screened against mammalian Cercopithecus aethiops (COS7) cells.

Pharmacophore modelling, X-ray crystallographic investigation of 3,4-dihydroxyphenyl derivative, and molecular docking studies of 3,4-dihydroxyphenyl derivative/ LdTop1-DNA ternary complex was then followed in order to attend optimum LdTop1 inhibitory activity.

Brindisi et al. reported for the first time that the couple tryparedoxin/tryparedoxin peroxidase is essential for parasite survival in the host since it neutralizes the hydrogen peroxide produced by macrophages during the infection [54].

The above paper describes the first noncovalent inhibitors of tryparedoxin peroxidase I of *Leishmania* parasite. The family of N,N-disubstituted 3-aminomethyl quinolone derivatives were developed by a comprehensive approach encompassing X-ray structure determination and a combination of High-Throughput Docking and preliminary hit optimization.

The most effective compound in both the HRP (horseradish peroxidase)-H2O2 competition assay and SPR (Surface Plasmon Resonance) experiments showed a binding mode able to form a H-bond with the backbone of His169, preserving the H-bond interactions. The fluorophenyl ring with an adamantyl moiety resulted in the formation of optimized hydrophobic contacts with Val51, Val128, Ile78 Pro186, Pro188, Pro53 and Phe50.

2.2 Ligand Based rational design

Recently some studies have been reported in which they are used effectively Quantitative Structure-Activity Relationship (QSAR) and Quantitative Structure- Property Relationship (QSPR) models to predict activity and ADMET parameters and the search for novel compounds via ligand-based virtual screening (LBVS). In one of the above studies Armitage et al. [55] based on the following design:

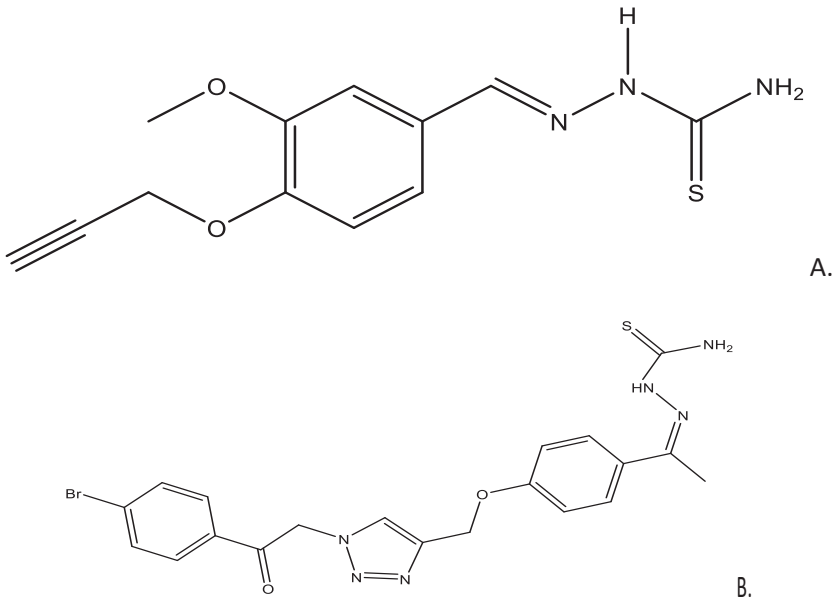


Figure 6. *Thiosemicarbazones derivative (A) and 1,2,3-triazole derivative (B) against both promastigotes and amastigotes of L. Major.*

***Leishmania* box → Study of metabolic action → Compound clustering**

Subsequently they applied capillary electrophoresis–mass spectrometry to identify the metabolic profile of *Leishmania donovani*, and principal components analysis was used to cluster compounds on potential mode of action.

Bhagat et al.[56] synthesized α-aminophosphonates derivatives following a modified Kabachnik–Fields reaction performed under solvent- and catalyst-free conditions at room temperature. Twenty six a-aminophosphonates were subjected to in vitro evaluation for anti-*leishmanial* activity against *L. donovani* promastigotes using the (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Seven of them **(Table 6)** displayed potent inhibitory activity. To establish the 3D structure–activity relationship, the above models were derived using the (R)- and (S)- stereoisomeric/enantiomeric forms of the a-aminophosphonate. The (S)-form generated the best predictive model.

Ashok et al. [57] studied the anti-*leishmanial* activity of tetrahydro-b-carboline derivatives which have recently been reported against both promastigote and amastigote forms of *L. infantum*.

Two of the sixteen derivatives which isolated and studied are listed in **Table 7**, exhibited selective and potent inhibition of amastigotes with IC50 values 0.67 and 0.87 mM respectively and potency was comparable with amphotericin B. It seems that substitution on meta, ortho positions showed favorable effect, while replacement with bulkier group had minimal effect on activity and para substitution was not desirable [57].

Temraz et al. identified new hybrid structures of 1,2,3-triazoles and thiosemicarbazones derivatives as novel anti-leishmanial agents. Most of the synthesized hybrids showed superior anti-leishmanial activity relatively to miltefosine, against both promastigotes and amastigotes of L. Major.

Compounds A and B **(figure 6)** showed nanomolar activity against promastigotes (IC50s are 227.4 nM and 140.3 nM respectively). Their IC50s against amastigotes were 1.4 μM and 1 μM respectively reaching 6 and 8 times the activity of miltefosine, respectively [58].

Da Trindade Granato et al. **59**, investigated the antileishmanial activity and cytotoxicity of novel steroids against murine macrophages. Among the sixteen derivatives, schiffbase2, Cholesterol Derivatives (CD2) and deoxycholic acid derivatives (DOCADs) were effective against promastigotes of *Leishmania species*. The absence of hydroxyl in the C-7 position of the steroid nucleus, as well as the modification of the acid group in DOCADs were considered important for antileishmanial activity.

Treatment of *L. amazonensis* promastigote forms with DOCAD5 induced biochemical changes such as depolarization of the mitochondrial membrane potential, increased ROS production and cell cycle arrest.

3. Conclusions

It is constructive to mention that the scientific data described in the review article confirm the satisfactory integration of computational and experimental strategies in the R & D of *leishmaniasis* drugs and that the global scientific community is moving in this direction.

An important advance in the field of *leishmaniasis* would be the validation of a higher number of molecular targets and suitable ligands. Therefore, the aforementioned research effort related to Structure Based Drug Design (SBDD) and Ligand Based Drug Design (LBDD) developed in recent years should be intensively continue for the synthesis and development of optimized drugs for *leishmaniasis*. □

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ΦΑΡΜΑΚΕΥΤΙΚΗ, 33, IV, 2021 | 272-291 ΑΡΘΡΟ ΕΠΙΣΚΟΠΗΣΗΣ

PHARMAKEFTIKI, 33, IV, 2021 | 272-291 REVIEW ARTICLE

An Overview of Common Chemotherapeutics Used in COVID-19 Illness from Pharmaceutical Chemistry Point of View

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KEYWORDS:
COVID-19, Chemotherapeutics, Antibiotics, Steroids, Antivirals, Antiparasitics

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ABSTRACT

The outbreak of COVID-19 pandemic early last year posed a serious threat to the life of the people across the globe. The cause of the COVID-19 is a new Corona virus called SARS-CoV-2. The origin of this virus is not known to date. Since the virus was a new one, obviously there was no clue whatsoever to deal with the infection caused by this virus. To manage the symptoms of virus infection such as fever, headache, coughing, breathing problem, pneumonia, etc. and deal with any possible secondary infection different classes of chemotherapeutics are in clinical use. These include molecules belonging to the class of antibiotics, steroids, analgesics/antipyretics, antiviral, anti-parasitic, anticoagulants, and supplements like vitamin C, vitamin D3, and zinc, etc. The present article gives an overview of the four essential therapeutics used from the medicinal/pharmaceutical chemistry point of view. These are antibiotics, steroids, antivirals, and antiparasitic drugs.

Introduction

COVID-19 disease is an infectious disease caused by a newly discovered coronavirus SARS-CoV-2[1]. The origin of this virus is not known to date. Most common symptoms of COVID-19 disease are mild fever, sore throat, cough, fatigue, and mild to moderate respiratory problem. Many people may go asymptomatic as well. Older people, and those with underlying medical problems like cardiovascular diseases, diabetes, chronic respiratory disease, and cancer are more prone to develop serious illness. The WHO declared on 30th January 2020 that the outbreak of COVID-19 caused by SARS-CoV2 constitutes a Public Health Emergency of International Concern (PHEIC). COVID-19 was characterized as a pandemic on 11 March 2020[2].

Task force consisting of medical experts, scientists, bureaucrats, social activists, and other government dignitaries were constituted in most countries and policies were drafted to manage the disease. At the beginning, most developing countries didn't have facility to even diagnose the virus. So the first and foremost requirement was to develop diagnostic facilities. Lockdown was implemented in several countries to stop the spreading of virus and contain the disease. The lockdown also gave some time to make appropriate planning to fight the pandemic. In several countries, temporary hospitals were built overnights. Since it was a new

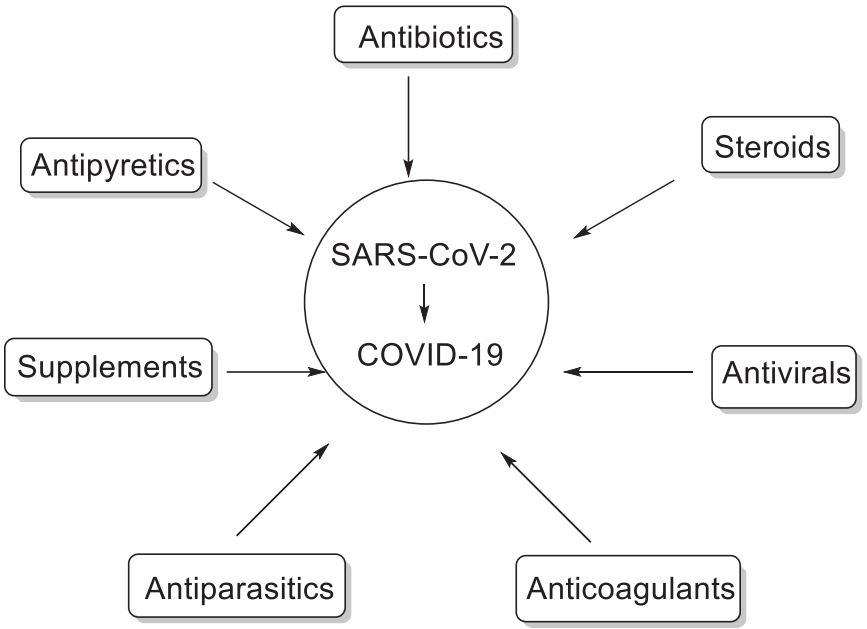


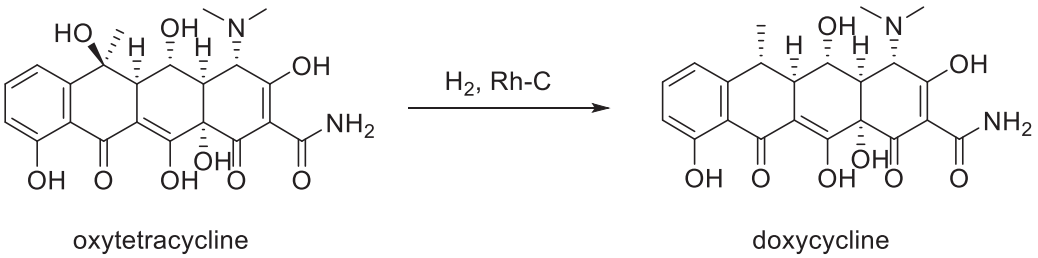
Figure 1. *Therapeutics used in COVID-19.*

virus, obviously there was no clue whatsoever to deal with the infection caused by this virus. To manage the symptoms of virus infection such as fever, headache, coughing, respiratory system problems, pneumonia, etc. and deal with any possible secondary infection different classes of chemotherapeutics have been recommended in different countries under the guidelines of the WHO and of their own expert panels. These chemotherapeutics include antibiotics, steroids, analgesics/antipyretics, antiviral, anti-parasitic, anticoagulants, and supplements like vitamin C, vitamin D3, and Zinc, etc. The supplements were recommended in 100% cases. There was generally a consensus on strengthening the immune systems and doctors recommended general public (whether infected with Corona virus or not) to take vitamin C, vitamin D3, and Zinc on daily basis.

The present article gives an account of chemotherapeutic such as antibiotics, steroids, antivirals and antiparasitics used in COVID-19 management (Fig. 1) from the medicinal chemistry point of view. This includes their discovery, structure, mode of action, approval, and their syntheses. The knowledge of the chemotherapeutics used is based on literature survey and author's interaction with over ten doctors and twenty five Covid-19 patients from some countries in Asia and Africa that include India, Pakistan, Bangladesh, South Africa, Zambia, Swaziland, and Botswana. The supplements used (vitamin C, zinc, and vitamin D3), antipyretics (paracetamol, and anticoagulant (usually aspirin) are too common molecules and well-known hence not discussed.

Antibiotics in covid-19 management

The most common antibiotics used in the countries mentioned above are either macrolides or β-lactam antibiotics. Azithromycin is the most commonly prescribed macrolide antibiotic for COVID-19 patients with mild symptoms[3]. β-Lactam antibiotics have been used in severe to critical cases and include penicillin (amoxicillin) in combination with clavulanic acid (β-lactamase inhibitor), cephalosporins (ceftriaxone, ceftazidime/cefepime, cefpodoxime), and carbapenems (meropenem). Use of fluoroquinolone ciprofloxacin was very limited hence not discussed.



oxytetracycline doxycycline

Scheme 1. *Catalytic hydrogenation of oxytetracycline to doxycycline.*

Polyketides and Macrolide antibiotics

Macrolides are the subgroup of polyketides and constitute an important therapeutic class (Fig. 2). They act against community-acquired respiratory infections such as community-acquired pneumonia, acute bacterial exacerbations of chronic bronchitis, acute sinusitis and tonsillitis[4-6]. Erythromycin A is the principal representative of macrolides. It was first isolated from *Streptomyces erythreus* at the Lilly in 1952[6]. Erythromycin has a limited antibacterial spectrum and limited solubility in acidic medium.

Azithromycin (the first azalide antibiotic on the market) is a second generation macrolide which together with some other macrolides gradually replaced erythromycin A. Azithromycin has higher potency, broader spectrum of activity, improved physicochemical and pharmacokinetic profiles, and attenuated side effects[7]. However, similar to erythromycin A, the second generation macrolides also suffered poor activity against macrolide resistant pathogens.

The macrolide antibiotics act by blocking bacterial protein biosynthesis as a result of binding to the 23S ribosomal RNA of the 50S subunit and interfering with the elongation of nascent peptide chains during translation[8]. Located in domain V, near the peptyl transferase site, macrolide antibiotics obstruct the peptide exit tunnel without affecting peptidyl transferase activity.

All macrolide antibacterial agents approved or in trial phase for use in human being are manufactured by chemical modification of erythromycin. Azithromycin is prepared from erythromycin in four steps. Seiple and coworkers have reported a practical, fully synthetic route to obtain macrolide antibiotics by the convergent assembly of simple chemical building blocks, enabling the synthesis of diverse structures not accessible by traditional semisynthetic approaches[9].

Doxycycline, 4-dimethylamino-1, 4, 4a, 5, 5a, 6, 11, 12a-oxtahydro-3, 5, 10, 12, 12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacencarboxamide is a broad-spectrum polyketide antibiotic used in the treatment of infections caused by bacteria and certain parasites. It is an isomer of tetracycline; the only difference being the hydroxyl group at C-6 in tetracycline is present at C-5 in doxycycline. Like other agents of the tetracycline class, it either retards the growth of bacteria or kills bacteria by inhibiting protein production. Doxycycline is in commercial use since 1967. It is on the WHO list of essential medicines. Charest and coworkers have reported a convergent enantioselective route leading to the synthesis of structurally diverse 6-deoxytetracycline antibiotics[10]. The formation of doxycycline can also be achieved by reductive dehydroxylation of C-6 hydroxyl group in oxytetracycline (Scheme 1)[11].

Its mode of action involves inhibition of the synthesis of bacterial proteins by binding to the 30S ribosomal subunit. This prevents the binding of transfer RNA to messenger RNA at the ribosomal subunit meaning amino acids cannot be added to polypeptide chains and new proteins cannot be synthesized. This stops bacterial growth giving the immune system time to kill and remove the bacteria.

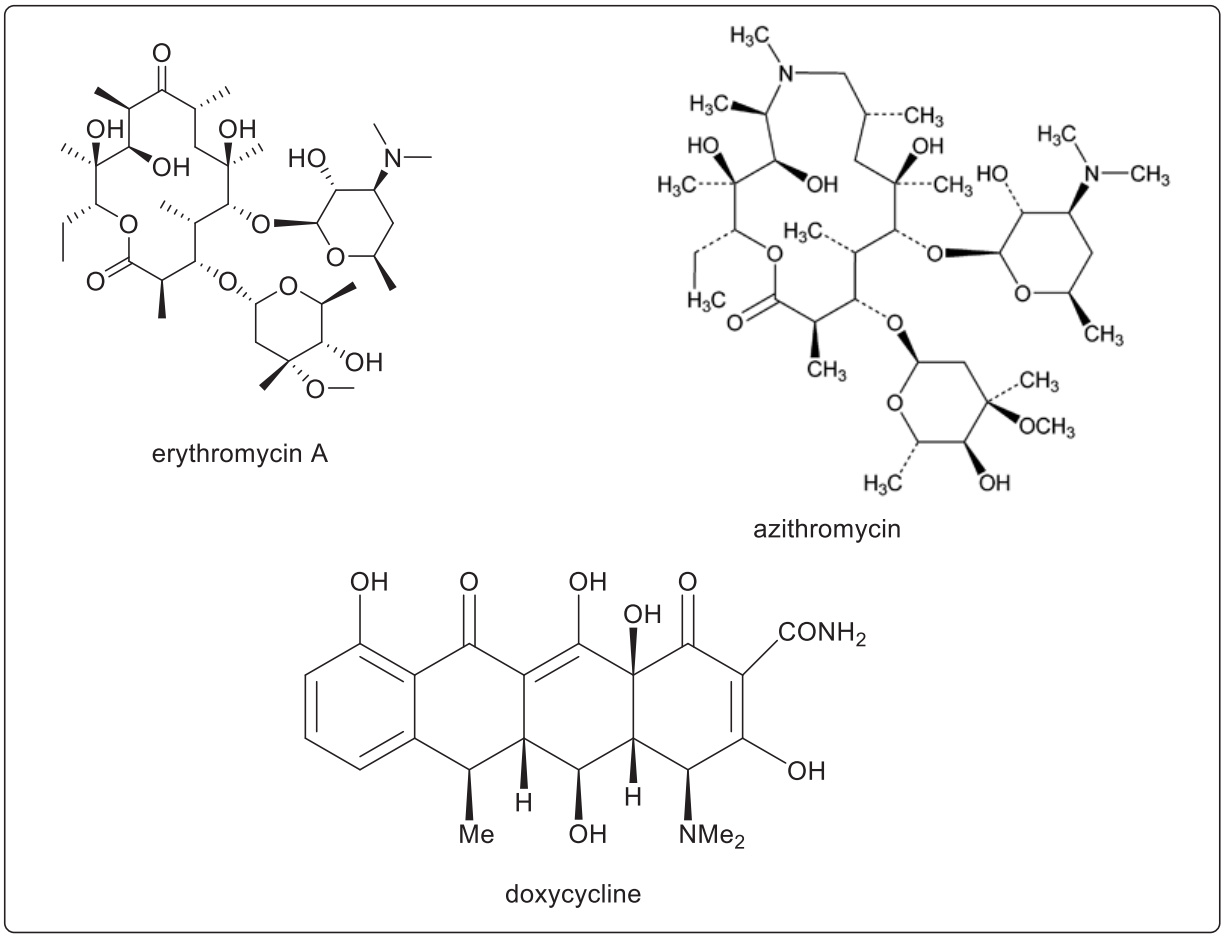


Figure 2. *Macrolide and ketolide antibiotics.*

β-Lactam Antibiotics and β-Lactamase Inhibitors

β-Lactams (Fig. 3) constitute the most common group of antibiotics in both human and veterinary medicine and share more than 65% of the world antibiotics market. They are also in clinical use as β-lactamase inhibitors. The core structure of the β-lactam antibiotics is the four-membered cyclic amides. The β-lactam ring may be fused to another heterocyclic ring such as substituted thiazolidine ring (penicillins), thiazine ring (cephalosporins), oxazolidine ring (clavulanic acid) or an azacycloalkyl ring (carbapenems)[12,13]. Clavulanic acid is used as β-lactamase inhibitor in combination with many other β-lactam antibiotics such as augmentin (combination of amoxicillin and clavulanic acid). The antibacterial activity mode of action of β-lactams involves inhibition of the bacterial cell wall synthesis because they can bind to the active site serine residue of the enzymes known as the penicillin-binding proteins (PBP), and thus inhibit synthesis of the peptidoglycan component of the bacterial cell walls[14].

β-Lactam antibiotics from all the subgroups mention above are getting prescribed for patients infected with SARS-CoV-2. The most common are penicillins-amoxicillin, cephalosporins-ceftriaxone, ceftazidime/cefepime, and cefpodoxime, and carbapenems- meropenem.

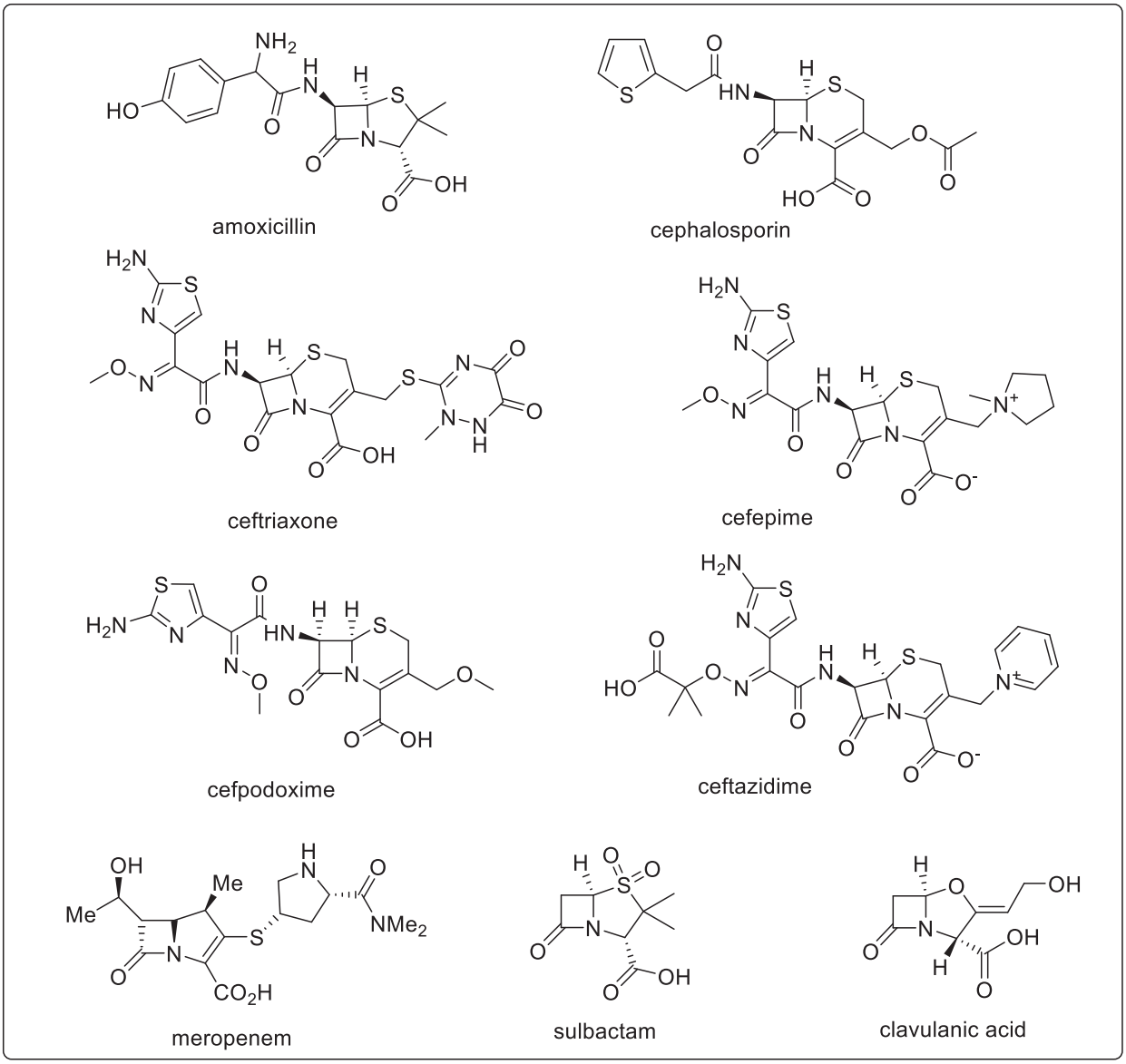
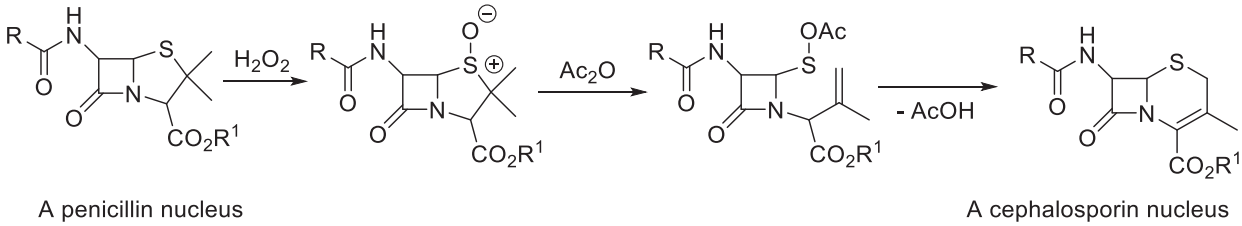


Figure 3. *β-Lactam antibiotics and β-lactamase inhibitors.*

Penicillin was first discovered by Fleming in the late 1920s and its structure was confirmed by X-ray crystallography in the late 1940s[15,16]. The synthesis of penicillins has been described long back by a convergent strategy[17]. This strategy involves, the synthesis of penicillamine and penilloaldehyde from alanine and phthalimide, respectively, followed by a series of reactions between the two to offer penicillins. Amoxicillin (α-amino-p-hydroxybenzyl penicillin) was discovered in 1958 and in clinical use since 1972. It is a semisynthetic derivative of penicillin with the core structure similar to ampicillin but with better absorption when taken by mouth. It is on essential medicines list of the WHO. The synthesis of amoxicillin is still an area of interest and several green methodologies using enzymatic reac-



Scheme 2. *Chemical transformation of penicillin to cephalosporin.*

tions have been reported for its synthesis in recent years[18,19].

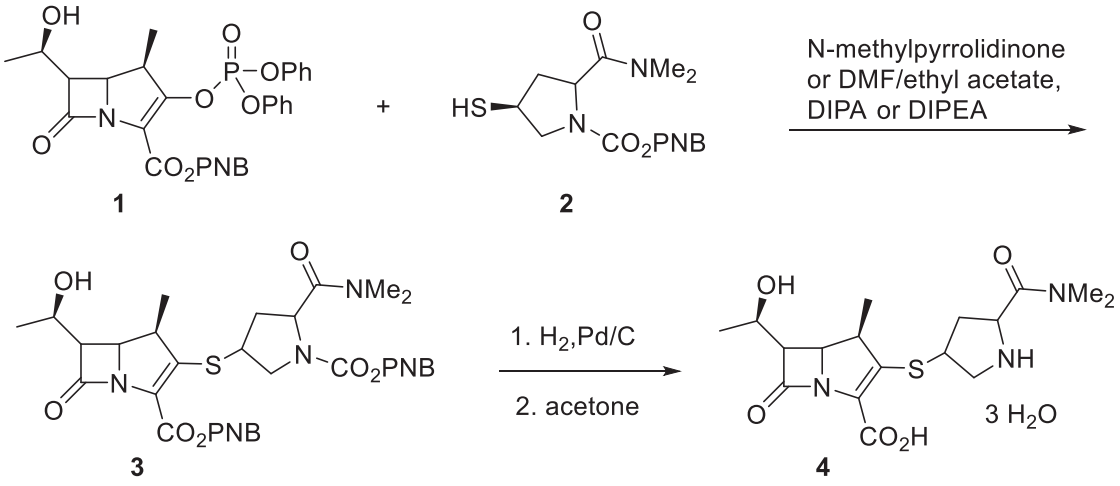
Cephalosporins also known as cephems are considered second generation β-lactam antibiotics. Cephalosporin compounds were first isolated from cultures of *Acremonium strictum* in 1948 by Italian scientist Giuseppe Brotzu[20]. Since then this group has seen a lot of development and now itself has entered into the fifth generation. Most of the cephalosporin antibiotics named above are either second or third generation cephalosporins. The first generation cephalosporins are active against a number of Gram-positive and Gram-negative bacteria but inactive against methicillin-resistant staphylococci. The second generation cephalosporins are less active against Gram-positive pathogens in comparison to the first generation cephalosporins but more active against Gram-negative pathogens. The third-generation cephalosporins are even less active against Gram-positive pathogens but have excellent activity against Gram-negative pathogens.

Cephalosporin can be easily accessed by simple chemical transformation of penicillin involving an oxidative enlargement of the thiazolidine ring system of penicillin to thiazine[21]. This has been realized by the following sequence of reactions, i.e., (a) oxidation of sulfur in thiazolidine ring, (b) the cleavage of the S-C2 bond, and (c) the cyclization to a six-membered dihydrothiazine ring with elimination of acetic acid (Scheme 2).

However, the investigations are in progress to synthesize new cephalosporins with broad activity. The main approaches in development of the new cephalosporin derivatives involve structural modifications at positions C-3 and C-7, and the development of cephem prodrugs. The compounds with a methoxy, carbamoyloxy or heteroaryl ring such as tetrazole or thiazole in the C-3 side chain are known to have potent antibacterial activity [22-26]. Recently, Jiang et al. have reported cephalosporin analogues with enhanced antibacterial activity by introducing a sulfamoylamino group[27].

Several carbapenem antibiotics have been developed since the discovery of thienamycin. Carbapenems have a broad antimicrobial spectrum and potent bactericidal activity. Meropenem is a β-methyl carbapenem. Meropenem has good stability to DHP-I due to steric hindrance of the β-methyl group at C-1 and an excellent spectrum against Gram-(-) bacteria. However, it is relatively less active against Gram-(+) bacteria. Meropenem was approved for clinical use in USA in 1996. It is on the list of essential medicines of WHO. Unlike other β-lactam antibiotics, it is highly resistant to degradation by β-lactamases.

The studies on carbapenems from a pharmaceutical point of view have been devoted mainly to the synthesis and evaluation of 1β-methylcarbapenem derivatives. The synthesis of meropenem was reported by Sunagawa et al. in 1990 [28]. The method involves condensation of commercially available 4-nitrobenzyl (4*R*,5*S*,6*S*)-3-[(diphenylphosphono)-oxy]-6-[(1R)-1-hydroxyethyl]-4-methyl-7-oxo-1-azabicyclo-[3,2,0]hept-2-ene-2-carboxylate(enol phosphate) 1 with (2S,4S)-2-dimethylaminocarbonyl-4-mercapto-1-(4-nitrobenzyloxycarbonyl) pyrrolidine (side chain) 2 in the presence of base to give diprotected meropenem 3. The latter compound on hydrogenolysis with Pd/C in the presence of buffer



Scheme 3. *Synthesis of meropenem.*

provides meropenem 4 (Scheme 3). This method was further improved by Tewari, et al [29].

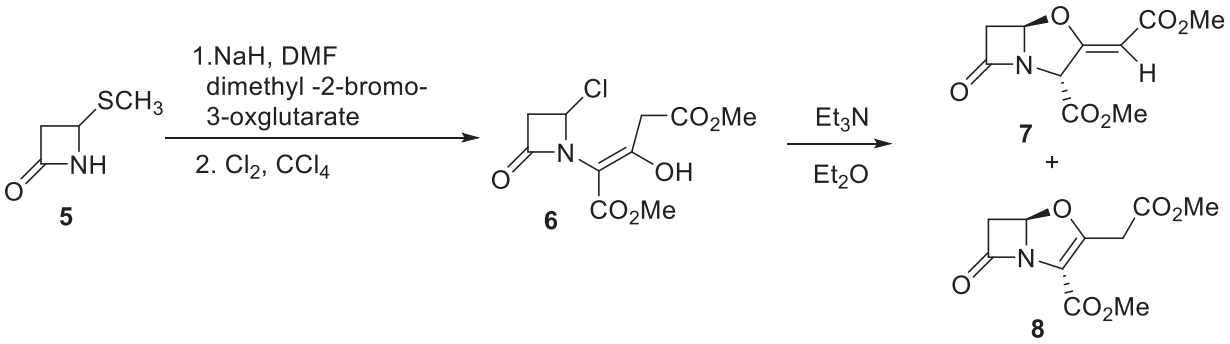
Clavulanic acid (Fig. 3) is a naturally occurring weak β-lactam antibiotic but is an excellent inhibitor of β-lactamase. It is produced by fermentation of *Streptomyces clavuligerus*[30]. It was first discovered in 1974-75 by the British scientists working at the drug company Beechum [31]. The molecule inhibits the activity of β-lactamase produced by many penicillin- and cephalosporin-resistant pathogens. Bentley et al. reported the first total synthesis of (±)-clavulanate in 1977 [32]. They reported the total syntheses of (±)-methyl clavulanates 7 and 8 starting from the 4-(methylthio)azetidin-2-one 5 via its alkylation with dimethyl 2-bromo-3-oxoglutarte followed by substitution of S-methyl group with a chloro group to form N-substituted azetidinone 6 which on cyclization with trimethylamine furnished the final product (Scheme 4).

Steroids in COVID-19 Management

After antibiotics, the most sought for compounds in managing the symptoms of COVID-19 are steroids. The WHO has recommended systemic corticosteroids for the treatment of patients with severe and critical COVID-19 [33]. The two most commonly used steroids are dexamethasone and methyl prednisolone (Fig. 4) [34]. The use of dexamethasone surged after June 2020 when preliminary data published suggested increased survival rate in in hospitalized patients with COVID-19 receiving oxygen or ventilator support.

Dexamethasone is similar to a natural hormone produced by adrenal glands. It is a derivative of hydrocortisone and an isomer of betamethasone. It is used to treat inflammation (swelling, heat, redness, and pain), certain forms of arthritis, severe allergies, asthma, and chronic obstructive lung disease. Dexamethasone is also used to treat certain types of cancer as well. As a glucocorticoid, dexamethasone is an agonist of the glucocorticoid receptor (GR). Its mechanism of action involves inhibition of cyclooxygenase-2 (COX-2), which is a key enzyme in the formation of eicosinoids. It is on the WHO's list of essential medicines. The synthesis of dexamethasone was first achieved in 1957 by Philip Showalter Hench and was approved for medical use in 1961 [35]. Arth and coworkers have synthesized dexamethasone from a multistep reaction starting from 16-pregnene-3-α-ol-11,12-dione acetate [36].

Recently, Dubashynskaya *et al.* have published a review describing the methods available for the synthesis of dexamethasone conjugates (carbodiimide chemistry, solid-phase synthesis, reversible addi-



Scheme 4. *Synthesis of clavulanic acid.*

tion fragmentation-chain transfer [RAFT] polymerization, click reactions, and 2-iminothiolane chemistry) and perspectives for their medical application as glucocorticoid drug or gene delivery systems for anti-tumor therapy. Additionally, the review focuses on the development of dexamethasone conjugates with different physical-chemical properties as successful delivery systems in the target organs such as eye, joint, kidney, and others [37].

Methyl prednisolone is a synthetic glucocorticoid primarily used as anti-inflammatory and immunosuppressive agent. It acts as a mineralocorticoid and glucocorticoid agonist. It was approved by the FDA in USA in 1957. Although methyl prednisolone is prescribed drug for the treatment of COVID-19 there are conflicting reports in literature about its safety profile or effectiveness [38]. The research are in progress to design and develop new methyl prednisolone conjugates with increased safety profile and improved activity [39]. Methyl prednisolone **13** is synthesized from hydrocortisone **9** (Scheme 5) [38]. The two keto groups of the latter are protected with ethylene glycol **10** in the presence of traces of acid to give protected hydrocortisone **11**. During the process, C=C at C4-C5 is shifted to C5-C6. Epoxidation of C=C with peroxybenzoic acid followed by treatment with methyl magnesium iodide introduces the methyl group on the C-6 position to furnish **12**. Reductive cleavage of the ketals followed by chemical and microbiological dehydration affords the methyl prednisolone.

Antivirals in COVID-19 Management

Since COVID-19 is due to the viral infection, the common sense suggests that antiviral compounds should be designed and developed for the treatment of COVID-19. It is also understandable to investigate the well-known antiviral agents for repurposing rather than dreaming of new molecules overnight. This section will discuss the development and use of remdesivir, favipiravir, and 2-deoxy-D-glucose in treating COVID-19.

Remdesivir was approved by the US FDA for the treatment of COVID-19 in October 2020 [40]. The panel had also recommended against the use of lopinavir/ritonavir, and other HIV protease inhibitors for the treatment of COVID-19 patients. Updated guidelines from the WHO in November 2020 included a conditional recommendation against the use of remdesivir for the treatment of COVID-19 [41]. However, remdesivir has been approved for emergency uses in over fifty countries. Remdesivir was originally developed for treating hepatitis C. It was then investigated for use in Ebola patients before being investigated for the treatment of COVID-19. Over ten countries including India and China have given approval to Favipiravir for use in COVID-19 patients. The discovery and development of remdesivir leading to its emergency use authorization for COVID-19 has been reviewed recently by Eastman, *et al* [42].

Remdesivir is a phosphiramidate prodrug of nucleotide capable to diffuse into cells, where it is con-

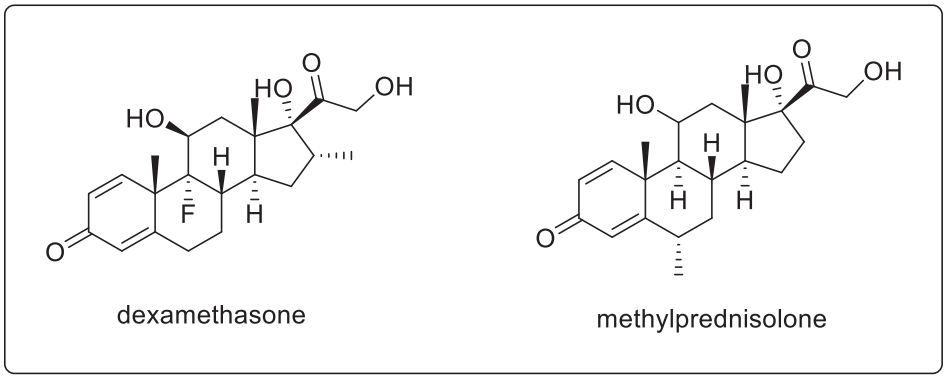


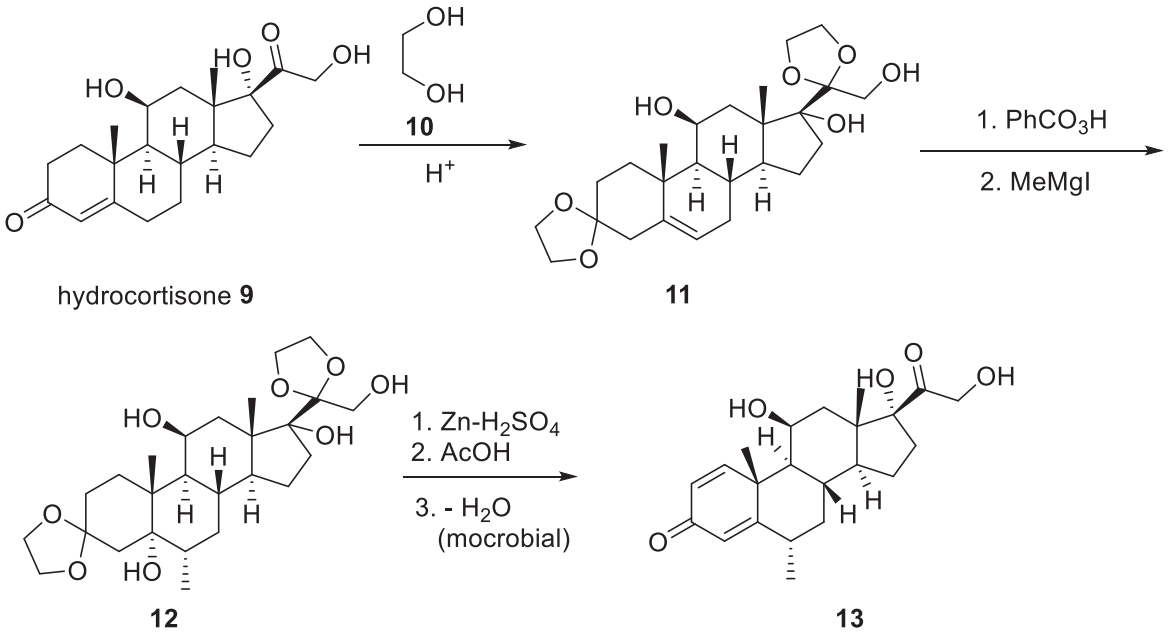
Figure 4. *Structures of dexamethasone and methylprednisolone.*

verted to GS-44152 monophosphate via the actions of esterases (SES1 and CTSA) and a phosphoamidase (HINT1); which in turn is further phosphorylated to its active metabolite triphosphate by nucleoside-phosphate. Although this bioactivation occurs in intracellular manner a substantial amount of remdesivir is prematurely hydrolyzed in plasma, with GS-441524 being the major metabolite in plasma. The active metabolite of remdesivir interferes with the action of viral RNA-dependent RNA polymerase leading to a decrease in viral RNA production [43].

The synthesis of remdesivir was reported by Chun *et al*. working for Gilead Sciences Inc [44]. This group employed a convergent strategy and prepared intermediates phosphoryl alaninate **17**, lactone **18**, and (4-aminopyrrolo[2,1-*f*] [1,2,4]triazin-7-yl)lithium **19** starting from alanine **14**, ribose **15**, and pyrrolo[2,1-f] [1,2,4]triazin-4-amine **16**, respectively. The reaction between **18** and **19** leads to the formation of alcohol **20** by nucleophilic addition of heteroaryl lithium on C=O of the lactone. The alcohol is then converted to nitrile **21**. At this stage of the synthesis, all the protecting groups are removed to give nucleoside **22** that reacts with intermediate **17** in the presence of trimethyl phosphate and methylimidazole to furnish a diastereomeric mixture of remdesivir that is resolved to give optically pure remdesivir **23** (Scheme 6).

With the outbreak of the COVID-19 pandemic, there is resurgence of interest in improving the synthetic methods to access remdesivir [45-47]. The synthesis of key intermediates have been investigated in order to improve the synthesis and stereochemical outcome. For example, choosing *p*-nitrophenolate precursor **24** instead of chloridate **17** generates a single Sp isomer **25** after crystallization (Scheme 7). This route is very useful in the stereoselective synthesis of the final product [48]. In a contribution from Kocienski, the scheme uses 4-amino-7-bromopyrrolo[2,1-*f*] [1,2,4] triazine to obtain the Grignard reagent by reaction with isopropyl magnesium chloride that is added on to the C=O of the lactone [49].

Xue *et al*. have reported an improved *C*-glycosylation step for the synthesis of remdesivir [50]. According to them, an efficient protection of the free amino group in pyrrolotriazine would be essential for the successful addition reaction of base to lactone**.** They observed that the addition of a secondary amine such as diisopropylamine was useful for improving the reaction efficiency. Wang *et al*. have reported a novel and efficient organocatalytic asymmetric synthesis of the remdesivir [51]. The chiral bicyclic imidazole was employed as the catalyst in coupling of the *P*-racemic phosphoryl chloride **26** with protected nucleoside **27** (GS441524) (Scheme 8). This unique chiral catalyst was very important for the dynamic kinetic asymmetric transformation to offer an excellent stereoselectivity (96% conv., 22 : 1 Sp : Rp). This group carried out 10-gram scale reaction of compounds **26** and **27** in the present of 10 mol % of effective chiral bicyclic imidazole catalyst **28** and 2,6-lutidine

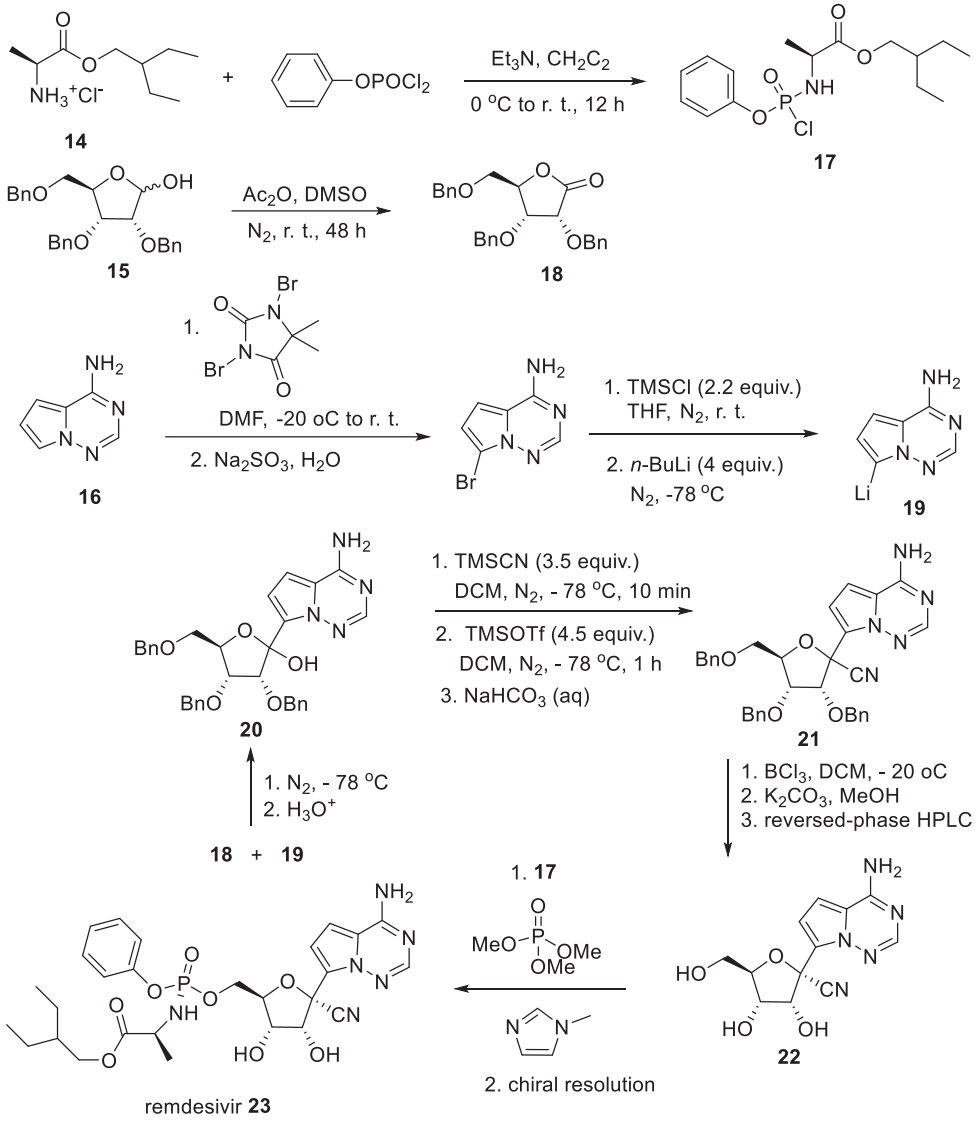


Scheme 5. *Synthesis of methylprednisolone from hydrocortisone.*

as a base in dichloromethane at –40 °C for 48 hours to furnish Sp remdesivir **29** with 96% conversion (22 : 1 Sp : Rp) [51]. Most recently, Gannedi et al. have reported this asymmetric organocatalytic reaction using 20 mol% of the chiral imidazole-cinnamaldehyde-derived carbamate as a catalyst. In this method, a mixture of (*S*)- and (*R*)-P-phosphoramidates were obtained in 97% yield (96.1/3.9 ratio). The catalyst was recovered in 83% yield for reuse, and similar result results were obtained [46].

Favipiravir, 3-hydroxy-6-fluoro-pyrazine-2-carboxamide, was in use to treat influenza in Japan. It has been authorized for treating COVID-19 in several countries including China, Japan, Russia, Turkey, and India, under emergency conditions. The mechanism of its actions is believed to be related to the selective inhibition of viral RNA-dependent RNA polymerase. Favipiravir is a prodrug that is metabolized to its active form, favipiravir-ribofuranosyl-5›-triphosphate (favipiravir-RTP) [52]. In recent years, investigation on favipiravir and its analogs as antiviral and antiparasite have drawn considerable interest [53-55].

Favipiravir **30** was first synthesized from methyl 3-amino-6-bromopyrazine-2-carboxylate **29** in five steps-diazotization-alcoholysis, Pd-catalyzed imine substitution/hydrolysis, aminolysis, Schiemann fluorination and demethylation (Scheme 9). In this method, some of the steps afforded poor yields and some used corrosive reagents and hence was not appropriate for scalable production. This method was improved by taking 3-hydroxypyrazine-2-carboxamide **31** which was transformed to 3,6-dichloropyrazine-2-carbonitrile **32** (Scheme 10). This dichloro-intermediate is then transformed to **33** by reacting with potassium fluoride, and subsequent nitrile hydration in concentrated hydrochloric acid or treatment with an alkaline solution of hydrogen peroxide to give **34**. The C3 fluorine of **34** is more reactive and could be easily replaced by hydroxyl group to furnish the final product. In the third method (Scheme 11) Liu *et al*. have prepared 3,6-dichloropyrazine-2-carbonitrile **32** by another method using cheaper 3-aminopyrazine-2-methyl-

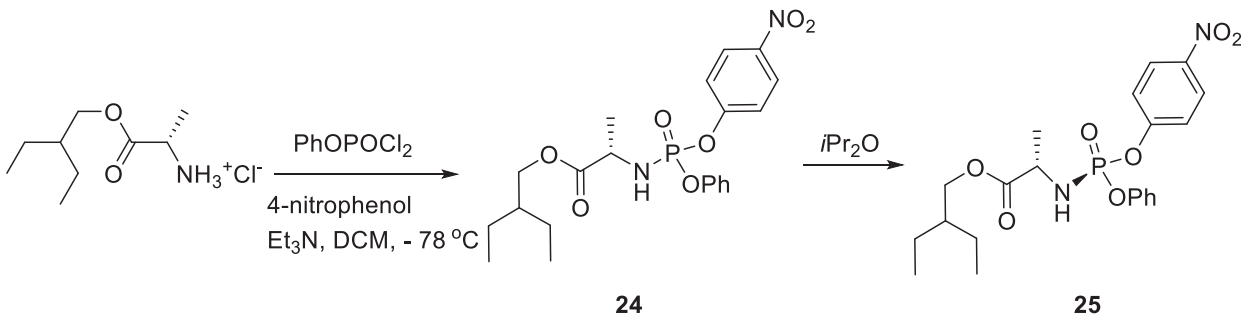


Scheme 6. *A convergent approach to the synthesis of remdesivir.*

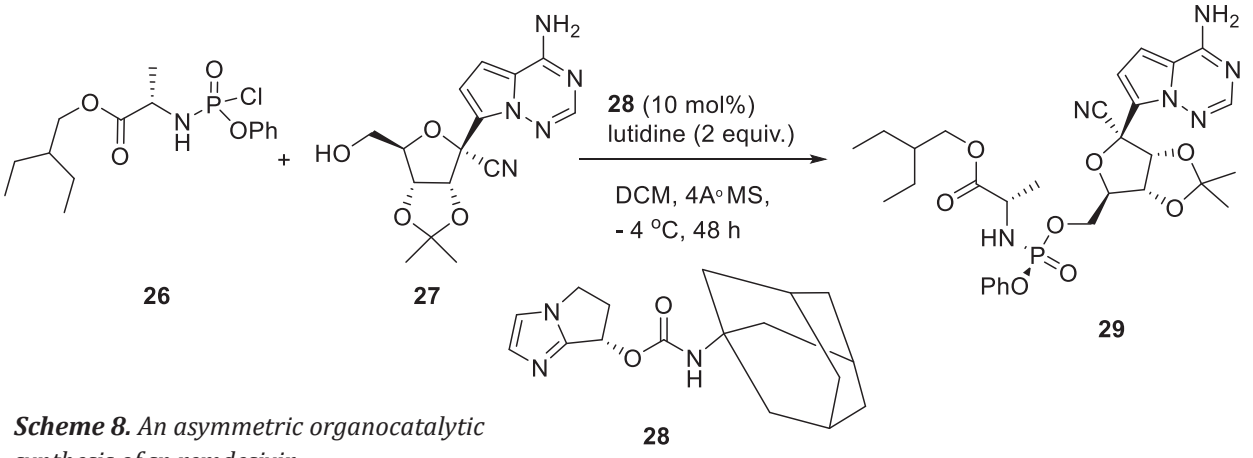
carboxylate **35** as the starting material to obtain **32** in 37% yields [55].

Recently, Guo *et al*. have reported the synthesis of favipiravir from an inexpensive and commercially available starting material 2-aminopyrazine **36** which was converted to 3,6-dichloropyrazine-2-carbonitrile **32** in good yield only in four steps avoiding the use of hazardous reagent $POCl_3$ (Scheme 12). In another three simple steps, the carbonitrile could be transformed into favipiravir [56].

2-Deoxy-D-glucose (Fig. 5), as the name indicates, is D-glucose analog having a hydrogen atom C-2 instead of a hydroxyl group. It has been studied in past as an anticancer agent and is in clinical trial. In May



Scheme 7. *Synthesis of an intermediate used in en route to remdesivir.*

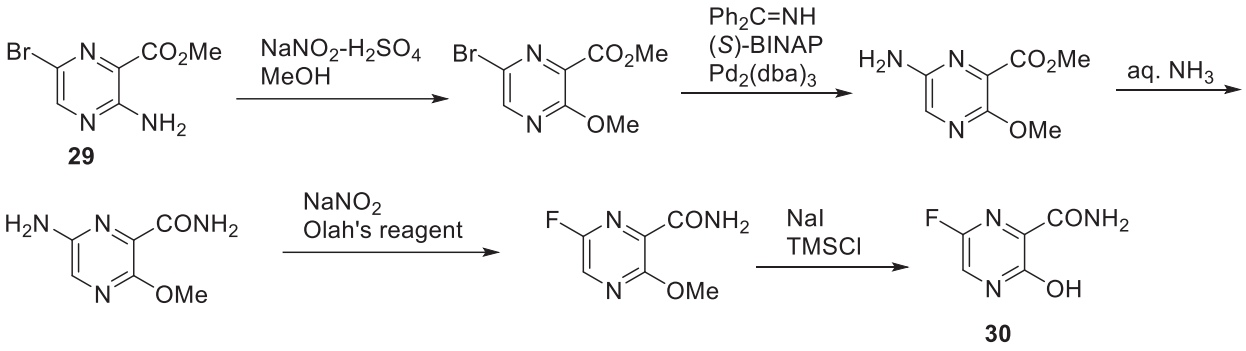


Scheme 8. *An asymmetric organocatalytic synthesis of sp remdesivir.*

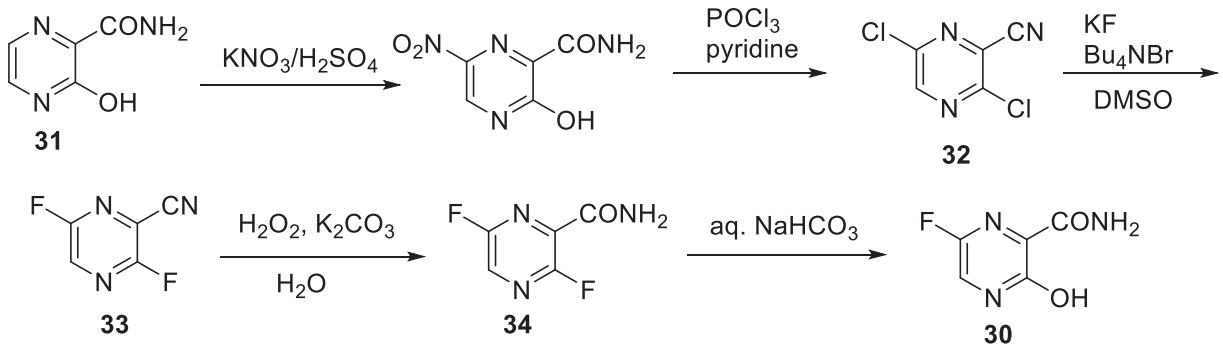
2021, the Drug controller General of India approved an oral formulation of 2-deoxy-D-glucose for emergency use as adjunct therapy in moderate to severe COVID-19 patients [57]. It was developed in collaboration between Institute of Nuclear Medicine and Allied Sciences (INMAS), India, Defense Research and Development Organization (DRDO), India, and Dr. Reddy's Laboratories, India. Although publication of phase 2 and phase 3 clinical trial data was pending at the time of approval it was said that the 2-DG reduced the supplemental oxygen dependence on COVID-19 infected patients and made their recovery faster [58].

Ghosh and Roy from Prescience Insilico Private Ltd. Bengaluru, and Singh from IIT Kanpur, India, have studied the possible inhibitory interaction of the drug with two different pathways (a) with non-structured viral proteins involved in translation and replication of SARS-CoV-2 and (b) its inhibition mechanism of the glycolysis pathway [59]. They have used automated novel drug designing platform with state-of-the-art free energy of binding calculator PRinMTML-ESS to study the role of 2-DG as an antiviral and glycolysis pathway inhibitor in SARS-CoV-2 affected humans. Based on docking, all atom molecular dynamic simulation and enhanced free energy sampling methods used in PRinMTML-ESS they have predicted that 2-DG effectively reduced the replication of SARS-CoV-2 in human cell by reducing the glycolytic flux, by competitive inhibition of glucose in binding with the enzyme hexokinase.

The application for patent of synthetic process to 2-deoxy-D-glucose starting from R-D-glucal was filed by Babu and Sreeman in 2003 [60]. This method involved haloalkoxylation of R-D-glucal wherein R was selected from H and 3,4,6-tri-*O*-benzyl to obtain alkyl 2-deoxy-2-halo-R-α/β-D-gluco/mannopyranoside. The latter was reduced to alkyl 2-de-



Scheme 9. *Synthesis of favipiravir from methyl 3-amino-6-bromopyrazine-2-carboxylate.*



Scheme 10. *An improved synthesis of favipiravir from 3-hydroxypyrazine-2-carboxamide.*

oxy- α/β-D-glucopyranoside which furnished 2-deoxy-D-glucose on hydrolysis.

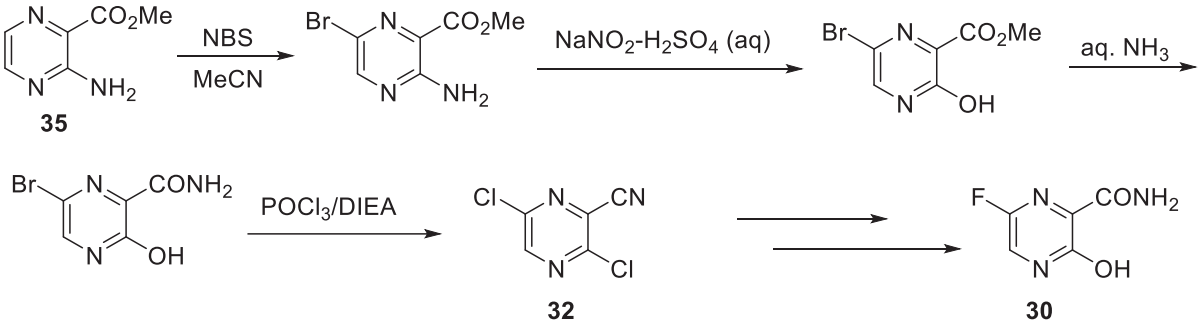
Anti-parasitic drugs in COVID-19 Management

The two much talked about and the most controversial anti-parasitic drugs during COVID-19 are hydroxychloroquine and ivermectin. These two drugs also drew the attention of the researchers most which resulted into plethora of literature on them. The objective here is not go into all those details but to know briefly about their historical development, current status in COVID-19 and synthetic methods.

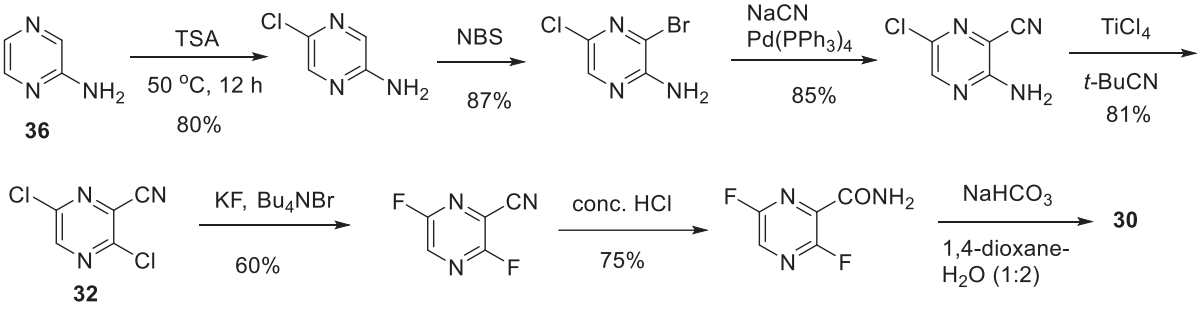
Hydroxychloroquine is a quinoline derivative. It is primarily used to treat malaria and rheumatoid arthritis. It was approved for medical use in USA in 1955 and is on the WHO list of essential medicines. As of now the clinical trials on its use in treating COVID-19 found it ineffective and it has been withdrawn from the treatment of COVID-19 [61].

An important mode of action of hydroxychloroquine is the interference of lysosomal activity and autophagy. It is widely accepted that hydroxychloroquine accumulate in lysosomes (lysosomotropism) and inhibits their function [62].

Yu *et al*. have recently reported a high yielding continuous-flow synthesis of hydroxychloroquine **44** starting with treatment of neat 3-acetyldihydrofuran-2(3*H*)-one **37** with HI to give 5-iodo-2-pentanone **38** [63]. The nucleophilic substitution of iodide **38** with aminoalcohol **39** gives compound **40** that is converted into oxime **41**. The oxime is reduced to amine **42** on treatment with hydrogen in the presence of Raney nickel. The reaction of the amine **42** with 4,7-dichloroquinoline **43** forms hydroxychloroquine **44** (Scheme 13).



Scheme 11. *Synthesis of 3,6-dichloropyrazine-2-carbonitrile from 3-aminopyrazine-2-methylcarboxylate.*



Scheme 12.

Avermectin family of compounds was isolated by Campbell and Omura from the culture broth of *Streptomyces avermitilis* b in 1975 [64]. It is widely used in low- and middle-income countries to treat worm infections. It is on the essential medicine list of the WHO. Since the start of the SARS-CoV-2 led pandemic, both observational and randomized studies have evaluated ivermectin as a treatment for, and as prophylaxis against, in COVID-19 infection. Several research papers and even review articles have appeared in literature on studies of ivermectin for use in COVID-19 [65]. Several studies have reported the effectiveness of ivermectin in treating COVID-19. For example, Caly *et al*. observed specific action of ivermectin against SARS-CoV-2 *in vitro* with a suggested host-directed mechanism of action being the blocking of the nuclear import of viral proteins that suppress normal immune responses [66]. The WHO, however, has recommended against its use outside the clinical trial. The National Institute of Health in the USA stated recently that there are insufficient data to recommend the use of ivermectin for treating COVID-19. The Indian government also removed ivermectin this year from the list of medicines to be used in treatment of COVID-19.

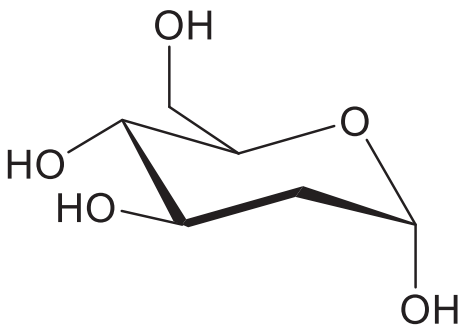
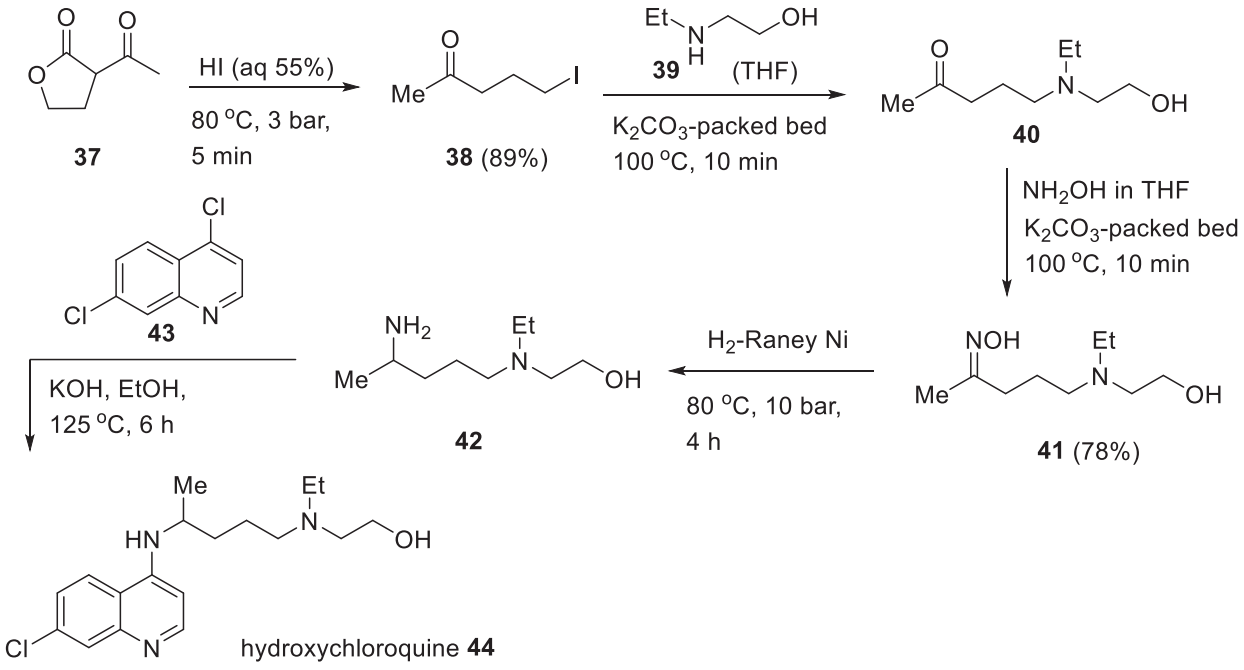


Figure 5. *Structure of 2-deoxy-D-glucose.*



Scheme 13. *Synthesis of hydroxychloroquine.*

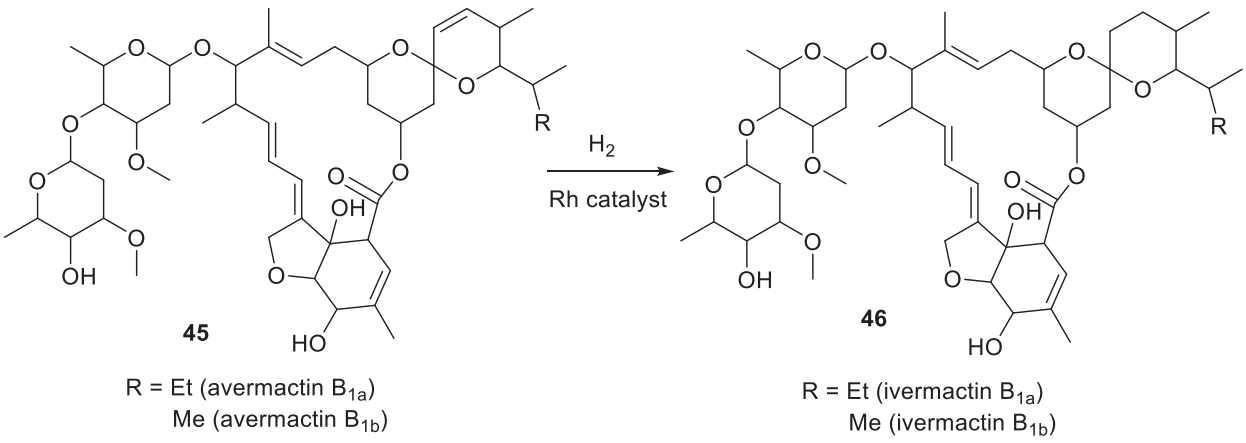
Ivermectin acts by interfering with nerve and muscle function of helminths and insects. The drug binds to glutamate-gated chloride channels that are common to invertebrate nerve and muscle cells, pushing these channels open. It results into an increased flow of chloride ions and hyper-polarization of the cell membranes. This hyperpolarization paralyzes the affected tissue, ultimately killing the invertebrate [67]

Fermentation of *Streptomyces avermitilis* yields eight closely related avermectin homologues, of which B1a and B1b form the bulk of the products isolated [68]. Due to their potential insecticidal and anthelmintic activities, as well as their unique pentacyclic architecture, the avermectin family attracted keen interest of synthetic organic chemists. The total syntheses for avermectin $B_{1a,}$ A1a, and ivermectin (aglycon) have been achieved and reported from time to time [69-71]. Recently, Yamashita *et al.* reported a more efficient and straightforward total synthesis of avermectin B1a, [72] as compared with previous syntheses.

The mixture of avermectins, in a separate chemical step, is selectively hydrogenated in the presence of Wilkinson's catalyst chlorotris(triphenylphosphine) rhodium(I) [RhCl(PPh3)3] to give ivermectin, which is an approximately 80:20 mixture of the two 22,23-dihydroavermectin compounds (Scheme 14) [73].

Conclusion

The four main classes of therapeutics used in managing the symptoms of COVID-19 are antibiotics, steroid, antiviral, and antiparasitic compounds. Macrolide and ketolide antibiotics are used in treating patients with mild symptoms. In severe cases, antibiotics from β-lactam group of antibiotics are in use. The mode of action of all these antibiotics are known. Recent literature shows interest in developing new methods for the synthesis of amoxicillin, and design and development of new cephalosporin and carbapenem antibiotics. Among steroids, investigations are on to develop new dexamethasone and methyl prednisolone an-



Scheme 14. *Catalytic hydrogenation of avermactin to ivermactin.*

alogues. When it comes to directly inhibit the viral multiplication, antiviral compounds are of great interest. Both the antiviral drugs – remdesivir and favipiravir have shown great promise. There is urgent need to give impetus to research on design and development of new antiviral molecules with selective activity against SARS-CoV-2. The well-known antiparasitic drugs like hydroxychloroquine and ivermectin appeared extremely useful in the beginning of the pandemic but no conclusive evidence of their usefulness could be obtained as is the case with supplements used. The research are still in progress to collect data on usefulness of these compounds in treating COVID-19. □

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ΦΑΡΜΑΚΕΥΤΙΚΗ, 33, IV, 2021 | 292-295 ΕΡΕΥΝΗΤΙΚΗ ΕΡΓΑΣΙΑ

PHARMAKEFTIKI, 33, IV, 2021 | 292-295 RESEARCH ARTICLE

Determination of the Concentration of Low Molecular Fraction of *Candida Albicans* Proteins by Elisa Method at Intramuscular Introduction in Candidiasis Therapy

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KEYWORDS:
candidiasis; low-molecular-weight proteins; antigen; vaccine; immunity; therapy

ABSTRACT

The purpose of this work was to determine the concentration of low-molecular fraction of C. albicans fungus protein at intramuscular introduction in therapy of candidiasis **Materials and methods.** The therapeutic effect of C. *albicans* fungus proteins in concentrations 1, 2, 3, 4, and 5 mg/mL has been examined in white mice. Animals were infected intraperitoneally with suspension of C. *albicans* fungus strain CCM 335-867 in the amount of 20 million cells per 1 mL volume. After 5 days and repeatedly after 14 days mice were injected intramuscularly in the upper part of the right hind paw with 0.2 mL of low molecular weight fraction of Candida fungus cell proteins. 14 days after each injection, the determination of the protective functions of the animal body has been carried out by the titer of specific C. albicans antibodies during immunoassay.

Results. According to the data obtained during studies on the treatment of candidiasis, it has been found that in the intramuscular route of administration after the first and second injection with a concentration of low molecular weight fraction of C. *albicans* 1, 2, 3, 4, or 5 mg/mL antibody titers increased, indicating that there is no activation of the protective functions of the body.

Conclusion. Proteins of low molecular weight fraction of C. *albicans* fungi with concentrations 1, 2, 3, 4, and 5 mg/mL do not activate the body's defense mechanisms

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1. Introduction

In recent years, the number of patients with candidiasis has increased dramatically, which is associated with the misuse of antibiotics, hormones, steroid drugs, as well as environmental degradation, increased pathogenicity of fungi of the genus Candida, and the development of comorbidities[1]. Candidiasis manifests itself in various forms, of which systemic and visceral candidiasis pose the greatest danger. These forms of candidiasis are characterized by a long course of the disease, have a variety of clinical manifestations, and frequent recurrences. Such forms of

candidiasis are difficult to treat with modern medicines, including antifungal antibiotics[2-3].

Adhesion of the Candida genus fungi is due to their ability to cleave secretory immunoglobulin A and α-antitrypsin and attach the fungus to mucosal proteins at the expense of the glycoprotein adhesion of the fungus' cell membrane. Adhesion of the fungus to the epithelium is the basis of its invasion in the body, which occurs in the first minutes of its interaction with the mucous membranes. The degree of adhesion determines the degree of C. *albicans* colonization, and the degree of invasion - its virulence. The fungi produce endotoxins, hemolysins, dermo-toxins, pyrogens, proteolytic enzymes that facilitate the adhesion of fungal cells to the cornified epithelium and mucous membranes. It has been established that in different strains of C. albicans the ability to produce these "factors of aggression", colonization and invasion are expressed to varying degrees, which should be taken into account when treatment is being prescribed[1, 4]. In the treatment of candidiasis, it is necessary to determine the type of pathogen and its sensitivity to antifungal drugs, especially in the treatment of various forms of invasive candidiasis, candidemia, and acute disseminated candidiasis - severe forms of candida infection with high mortality. After determining the type of Candida fungus, the antibiotic to which the pathogen is sensitive should be determined, and the dose and duration of therapy should be adjusted[4-6].

However, in the current practice doctors in Ukraine very rarely identify the type of Candida fungus, which is the causative agent of the disease. The most commonly diagnosed is candidiasis caused by fungi of the genus Candida without specifying the species. It should also be noted that even with a will to identify the type of Candida fungus, it is not always possible to do so due to limited reagents, equipment, and work experience of laboratory staff. In this situation, doctors prescribe antifungal therapy according to the standard scheme, using, most often, amphotericin B or fluconazole, even though the causative agents of this disease are not sensitive to these drugs. Also, given the long-term use of the same antifungal agents, many species of Candida fungi or their individual epitopes have lost sensitivity to them[4, 5, 7].

Many researchers believe that the use of drugs that can stimulate protective immune responses against candidiasis, i.e. immunobiological drugs that stimulate anti-fungal functions, is a promising direction in the fight against candidiasis[3, 7-9] and is an alternative to antifungal drugs. Various researchers have suggested several vaccine variants [2, 4, 5], but there is no consensus among them. One variety is subunit vaccines. They consist of fragments of antigens capable of providing an adequate immune response. These vaccines can be presented as both microbial particles or obtained in a laboratory using genetic engineering technologies.

Based at the Biotechnology and Microbiology, Virology and Immunology Department of the National University of Pharmacy, *Kharkiv, Ukraine,* authors have developed a method for the disintegration of Candida fungal cells using ultrasonic radiation. The composition of the extract-disintegrate of Candida cells includes proteins and polysaccharides that possess antigenic properties. In this case, according to the requirements of the State Pharmacopoeia of Ukraine, the identification of the active substance is carried out in terms of protein.

Previously, studies were conducted to determine the effectiveness of subcutaneous and intramuscular injections of C. albicans fungus cells disintegrate solution with a molecular mass of antigens greater than 10 kDa in animal experiments for prevention and treatment of candidiasis infection.[10, 11] The studies have found that the antigens of this fraction show immunogenic properties at C. albicans fungus proteins concentration of 3 mg/mL at intramuscular administration and do not exhibit immunogenic properties at subcutaneous administration. It is interested to conduct a study of low molecular weight fractions less than 10 kDa with C. albicans protein concentrations of 1, 2, 3, 4, and 5 mg/mL for immunogenicity at subcutaneous and intramuscular injection by antibody titers in the prevention and treatment of candidiasis.

The purpose of the work was to determine the concentration of low-molecular fraction of C. albicans fungus protein at intramuscular introduction in therapy of candidiasis.

2. Materials and methods

The study used strain CCM 335-867 of C. albicans fungi. To obtain the biomass of C. albicans fungi, the culture was cultivated. C. albicans fungi were first seeded in tubes on Saburaud agar medium using a sterile swab in a laminar box maintaining aseptic conditions. Cultiva-

Table 1: Therapeutic effect of C. albicans fungal cell antigens

Animals	C. albicans protein content, mg/mL	Administration Method	C. albicans AB titers in ELISA		
			healthy animals	ill after 1st injection	ill after 2nd injection
Mice	1	i.m.	1: (200 ± 10)	1: (400 ± 18)	1: (400 ± 28)
Mice	2	i.m.	1: (300 ± 14)	1: (800 ± 34)	1: (800 ± 35)
Mice	3	i.m.	1: (200 ± 9)	1: (600 ± 27)	1: (400 ± 18)
Mice	4	i.m.	1: (400 ± 17)	1: (600 ± 18)	1: (600 ± 25)
Mice	5	i.m.	1: (300 ± 13)	1: (800 ± 28)	1: (800 ± 37)

Note: n = 10

tion of C. albicans fungi in test tubes was performed for 48 hours maintaining a temperature of 25 ± 2 ºC. The microbiological purity of C. albicans fungi culture was checked visually and by microscopy. Next, the cells of C. *albicans* fungi were washed with sterile isotonic saline solution. The resulting suspensions of C. *albicans* fungal cells were seeded on Saburaud agar medium in mattresses and cultured for six days. The temperature was maintained at 25 ± 2 ºC throughout cultivation. The microbiological purity of C. *albicans* fungi culture was checked visually and by microscopy. Next, the cells of C. albicans fungi were washed with sterile isotonic saline solution. To separate the C. albicans fungi cells from saline solution, the suspension was centrifuged. During centrifugation, the rotational speed was 3000 rpm. The process lasted ten minutes. Next, the cells of C. *albicans* fungi were standardized to $9x10^8$/mL and destroyed by ultrasound. During the ultrasonic destruction of C. albicans fungal cells maintained a temperature of 25 ± 2 ºC, a frequency of 22 kHz, an intensity of 5 W/cm2. The process lasted fifteen minutes. Next, filtration was performed to separate biological material with a size of 10 kD. Determination of protein content was performed according to the State Pharmacopoeia of Ukraine. Next, pre-filtering with throughput capacity of 0,45 μm and sequentially sterilizing filtering with throughput capacity of 0.22 μm were performed.

The studies used the protein concentrations of C. albicans 1, 2, 3, 4, and 5 mg/mL. The study was performed on white mice. In both, control and experimental groups, 10 animals were used. The studies were conducted at the State Institution "II Mechnikov Institute of Microbiology and Immunology", Kharkiv, Ukraine. To determine the effectiveness of experimental concentrations of C. *albicans* fungus proteins it was necessary to carry out infection of white mice with the pathogen. To do this, used a C. *albicans* fungi suspension of strain CCM 335-867. Mice were infected intraperitoneally with a suspension of C. *albicans* at a dose of 20 million/mL. Five days after infection, mice were injected intramuscularly with 0.2 mL of Candida proteins. Mice in the control group were injected saline. 14 days later, the titers of specific antibodies to C. albicans were determined. For this, an enzyme-linked immunosorbent assay was used to detect G antibodies to C. albicans using the Vector-Best ELISA test system. Then, 14 days after the first injection, injected the second 0.2 mL injection of Candida cell proteins. Then, 14 days after the second injection, the titers of specific antibodies to C. albicans were determined and the results were analyzed.

All animal experiments were carried out following the EU Directive 2010/63/EU for animal experiments.

The calculation of antibody titers was performed according to the requirements of the State Pharmacopoeia of Ukraine "Statistical analysis of results of biological tests and quantitative determinations" Vol. 1 of the State Pharmacopoeia of Ukraine 2nd edition (SPU 2.0).

3. Results and discussion

Studies have shown the value of specific G antibodies to C. *albicans* in healthy mice, sick mice immunized with one injection, and sick mice immunized with two injections. Studies have shown that the titers of specific antibodies

to C. *albicans* fungus in healthy mice were in the range of 1:200-1:400. It should be noted that C. albicans fungi are part of the normal microflora of animals and they can be carriers of this fungi. Mice are also likely to come into contact with C. *albicans* fungus in the course of life, which is subsequently reflected in the titers of specific G antibodies to C. albicans fungi.

All studied concentrations of the low molecular weight fraction of C. *albicans* at immunization of mice with the first injection have demonstrated the formation of insignificant protective functions in animals. This is confirmed by the growth in titers of specific G antibodies to C. *albicans* fungi as compared to the titers values in healthy animals. The results of the studies are summarized in Table 1.

Studies have shown that after the second intramuscular injection of the low molecular weight fraction of C. *albicans* at concentrations of 1, 2, 3, 4, and 5 mg/mL, no sufficient protective immunity is formed in animals. This is evidenced by the fact that in all cases there was no increase in the titers of specific G antibodies to C. *albicans* fungus as compared to those after the first injection.

The titers of specific G antibodies to C. *albicans* fungus in the control group at the intramuscular route of administration of the low molecular weight fraction of C. albicans did not grow.

The data obtained from the research indicate that the low molecular weight fraction of C. *albicans* in concentrations of 1, 2, 3, 4, and 5 mg/mL after the first intramuscular injection provides only a minor activation of protective functions as evidenced by the two-fold increase in the concentration of C. *albicans* fungi antibodies. After the second injection of the low-molecular-weight fraction of C. *albicans* at the studied concentrations for further activation of protective functions, C. *albicans* fungi antibodies should increase several more times. However, this is not observed, which testifies of the absence of the required protective immunity to candidiasis.

4. Conclusion

The data obtained during the studies indicate that the low molecular weight fraction of C. albicans at concentrations of 1, 2, 3, 4, and 5 mg/mL at intramuscular injection of 0.2 ml of test specimens with an interval of 14 days provide only a two-fold increase of specific G antibodies titers to C. *albicans* fungus. This testifies of insufficient formation of protective immunity to candidiasis. Thus, the low molecular weight fraction of C. albicans cannot be used to develop a vaccine for the prevention and treatment of candidiasis. □

Declarations of interest: none

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ΦΑΡΜΑΚΕΥΤΙΚΗ, 33, IV, 2021 | 296-303 ΕΡΕΥΝΗΤΙΚΗ ΕΡΓΑΣΙΑ

PHARMAKEFTIKI, 33, IV, 2021 | 296-303 RESEARCH ARTICLE

Pharmacological Justification of the Active Substances in the Composition of Medicated Chewing Gum against the Background of Experimental Xerostomia in Rats

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KEYWORDS:
Xerostomia; medicated chewing gum; lysozyme hydrochloride; ascorbic acid; salivary glands

ABSTRACT

Xerostomia is a quite common pathological condition, in which the protective mechanisms of the oral cavity and the organism as a whole are suppressed. The current therapeutic management of xerostomia is primarily aimed at exogenous moisturing of the oral mucosa and stimulating saliva production. According to the literature, the use of chewing gums in patients with "dry mouth" syndrome contributes to a significant increase in salivation not only due to mechanical stimulation, but also due to the presence of various ingredients in their composition that affect the taste buds of the oral cavity. The aim of the study was to pharmacologically evaluate the effect of the composition of active pharmaceutical ingredients – lysozyme hydrochloride and ascorbic acid in compressed medicated chewing gum on salivation parameters and functional status of salivary glands of rats with experimental atropine-induced xerostomia. The effect was determined both for each active substance separately and for their combination. It was found that irrigation of the oral cavity of rats with experimental xerostomia with a combined solution of the composition of lysozyme hydrochloride and ascorbic acid at a dose of 4 and 8 mg/kg, respectively, for 14 days, led to a correction of spontaneous salivation rate and antioxidant status of the submandibular salivary gland tissues compared to the level of intact animals. In addition, the use of the test composition did not lead to changes in the pH of saliva, which is an additional factor of the good safety profile.

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Introduction

Xerostomia or "sensation of oral dryness" is a fairly common pathological condition that can be caused by a number of different factors: infectious and inflammatory diseases of the oral cavity, chronic systemic diseases, drugs medication, radiation and chemotherapy in the process treatment of cancer, congenital pathologies of the salivary glands, prolonged high psycho-emotional stress, smoking, improper or lack of hygienic procedures, deficiency of vitamins and microelements in the diet, lack of chewing load, etc.[1-5]

Against the background of hyposalivation, the protective mechanisms of the oral cavity and the organism as a whole are suppressed[6,7], which, accordingly, requires timely and effective treatment-and-prophylactic measures. The current strategy of xerostomia therapy is primarily aimed at exogenous moisturizing the oral mucosa and stimulating saliva production.[4,8-10]

According to the literature, the use of chewing gum in patients with "dry mouth" syndrome contributes to a significant activation of salivation not only due to mechanical stimulation, but also due to the presence of various ingredients in their composition that stimulate the salivary glands.[4,9-11] Substances that can stimulate salivation include spices, mint and fruit compositions, organic acids (citric, ascorbic or malic).[10,12-15]

In order to moisturize the oral cavity, rinsing, irrigation and lotions with solutions of glycerin, lysozyme, as well as products based on lipids, peptides and polysaccharides of natural origin are usually used.[4,8,16,17]

This study concerned the experimental pharmacological study of a newly developed drug in the form of a compressed medicated chewing gum (MCG) without sugar, containing lysozyme hydrochloride (LH) and ascorbic acid (AsA) as active pharmaceutical ingredients (APIs).[18,19]

Given that chewing gum as a dosage form has certain limitations for pharmacological research, which is associated with the behavior of laboratory animals, the object of experimental study were the APIs which are in the composition of the drug. In accordance to the results extrapolated from biopharmaceutical studies of previously developed MCG[20], the application of the composition was performed in the form of oral cavity irrigation with an aqueous solution of APIs, which was equivalent to estimated dose from gum used. According to the results of the kinetics of drug release from the studied compressed MCGs by in vitro method (Ph.Eur. 9.0, chapter 2.9.25), the average percentage of release of AsA and LH from gum samples after 10 min was 93.34% and 95.83%, respectively, and after 30 minutes – 99.55% and 99.71%, respectively. Therefore, previous biopharmaceutical studies have shown almost complete release of APIs from developed MCGs.

Table 1. Description of APIs used in the study

Name of ingredient	Manufacturer	Amount, mg/ per gum
Lysozyme hydrochloride	Bouwhuis Enthoven B.V., Netherlands	10.0
Ascorbic acid	Foodchem International Corporation, China	20.0

The aim of the study was to evaluate the effect of the APIs on salivation parameters and functional status of salivary glands of rats with experimental atropine-induced xerostomia.

Materials and Methods

Material

APIs of medicated chewing gum: lysozyme hydrochloride and ascorbic acid in the form of a solution, which was the experimental equivalent of compressed MCG (Table 1).

Methodology of research

The experiment was planned according to the Fig. 1.

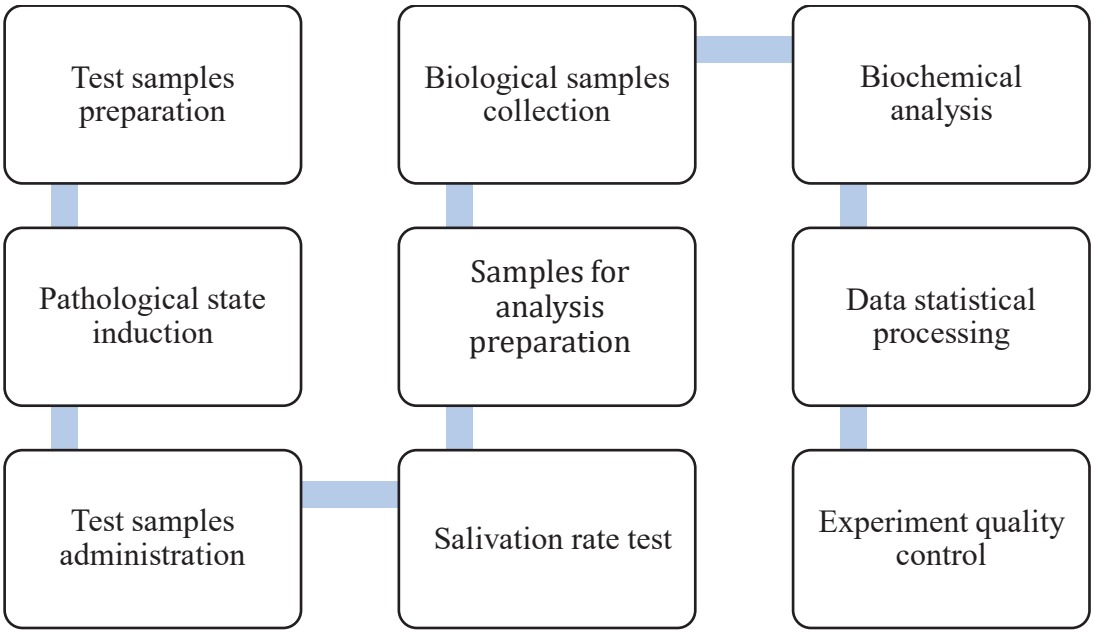

***Figure 1**: Design of experiment*

Animal maintenance and ethical approval

The study was performed on 40 outbred female rats aged 2.5-3 months. Healthy animals were selected for the experimental groups. The groups were formed by randomization through method of minimizing the difference in body weight.

The animals were kept in vivarium of Educational and Scientific Institute of Applied Pharmacy of the National University of Pharmacy (Kharkiv, Ukraine) under a 12:12 hour light/dark cycle, under constant temperature (22-24ºC) and were provided food and water *ad libitum*. The study was conducted in accordance with the Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes, directive of the European Parliament and of the Council of the EU 2010/63/EU of 22 September 2010 "On the protection of animals used for scientific purposes".[21] The experimental protocols were approved by the Bioethics Commission of the National University of Pharmacy (Approval No. 5 of 25 March 2021).

Experimental groups of animals

Experimental animals were divided into 5 experimental groups of 8 animals each:

1) Intact control – animals that have not been exposed by experimental pathology, irrigation of the oral cavity with solvent (IC).

2) Control pathology – animals that have been exposed by the model pathology of xerostomia, no treatment (CP).

3) Reference group №1 – animals that were irrigated with a solution of ascorbic acid (CP+AsA) on the background of experimental xerostomia.

4) Reference group №2 – animals that were irrigated with lysozyme solution (CP+LH) on the background of experimental xerostomia.

5) Test group – animals, which on the background of experimental xerostomia were irrigated with a combined solution of ascorbic acid and lysozyme hydrochloride (CP+AsA+LH).

Reproduction of experimental xerostomia, animal exposure scenarios and dosing criteria

Recreating the experimental xerostomia was per-

Table 2: Salivation parameters of rats with experimental xerostomia

Experimental group	Spontaneous salivation rate, ml / min	Salivary pH
IC	0.016 ± 0.001	8.11 ± 0.06
CP	0.006 ± 0.001*	7.80 ± 0.11
CP+AsA	0.007 ± 0.001*	7.66 ± 0.08*
CP+LH	0.007 ± 0.001*	7.90 ± 0.06
CP+AsA+LH	0.013 ± 0.001**/#/##	8.00 ± 0.07#

** – differences are significant relative to the corresponding group values IC, Tukey HSD test, p<0.05*

*** – differences are significant relative to the corresponding group values CP, Tukey HSD test, p<0.05*

 # – differences are significant relative to the corresponding group values CP+AsA, Tukey HSD test, p<0.05

– differences are significant relative to the corresponding group values CP+LH, Tukey HSD test, p<0.05

formed by applying to the oral cavity of rats 0.01% solution of atropine sulfate in an amount of 0.05ml (1 drop) using a microdose. The manipulation was performed daily on an empty stomach for 14 days.[22-24] During the entire period of atropine application the oral cavity was irrigated in the treatment-and-prophylactic mode with experimental samples or solvents (depending on the experimental group) once a day 2 hours after the application of atropine.

The study dose of active substances was recalculated from the recommended daily dose for clinical use through interspecific coefficients of difference in body weight and surface area.[25] Also, during dose extrapolation, the results of previous biopharmaceutical studies were taken into account.[20] Thus, it was determined that as an analogue of chewing gum in the experiment it was advisable to use a solution of lysozyme hydrochloride and ascorbic acid: 0.004 g/kg and 0.008 g/kg, respectively. In the reference groups, the animals received monotherapy equivalent to the dose of the corresponding active substance.

Sample preparation and analysis

After 2 weeks of treatment the animals' salivation was studied. The saliva secreted in animals during 30 minutes of anesthesia-induced sleep (thiopental, 40 mg/kg) was collected using a microdose to determine the gross rate of spontaneous salivation (ml/min).

After salivation parameters were determined, the animals were removed from the experiment by hypoxia in a CO_2 box and parotid, submandibular, and sublingual salivary glands were surgically removed.

From the fragments of the submandibular salivary glands, 10% homogenate was added in standard PBS-buffer[26] and a supernatant was obtained to determine the parameters of the pro-/antioxidant system. The content of thiobarbituric acid reactants, reduced glutathione and catalase activity were determined as markers of tissue oxidative status, which were determined according to standard routine methods[27]. Analysis for TBA reactants was performed by standard reaction with thiobarbituric acid, after precipitation of proteins with trichloroacetic acid, the measurement of the optical density of the sample is performed against the control sample at 532 nm. Determination of catalase activity was performed by a conventional method based on the reaction of formation of colored complexes of hydrogen peroxide and ammonium molybdate with an optimal absorption length of 410 nm. The reaction to detect reduced glutathione was based on the interaction of 5,5′-Dithiobis-nitrobenzoic acid (Ellman's reagent) and SH-substances; the optical density of the sample is performed against the blank at 412 nm.

Instrumentation and measurements

The pH of saliva obtained during the experiment was determined using a pH meter "pH-150 MI" (LLC "Spectro Lab", Russia). Mass coefficients of salivary glands were measured using laboratory scales "ADS 200" (LLC "AXIS", Ukraine). Quantitative determina-

Table 3: Mass coefficients of salivary glands of rats with experimental xerostomia

Experimental group	Mass coefficients of salivary glands		
	Parotid, g/100 g	Submandibular, g/100 g	Sublingual, g/100 g
IC	0.221 ± 0.011	0.181 ± 0.009	0.046 ± 0.005
CP	0.276 ± 0.012*	0.249 ± 0.009*	0.058 ± 0.005
CP+AsA	0.268 ± 0.006*	0.241 ± 0.005*	0.055 ± 0.004
CP+LH	0.255 ± 0.006	0.236 ± 0.006*	0.053 ± 0.005
CP+AsA+LH	0.240 ± 0.006**	0.204 ± 0.008**/#/##	0.050 ± 0.005

** – differences are significant relative to the corresponding group values IC, Tukey HSD test, p<0.05*

*** – differences are significant relative to the corresponding group values CP, Tukey HSD test, p<0.05*

 # – differences are significant relative to the corresponding group values CP+AsA, Tukey HSD test, p<0.05

– differences are significant relative to the corresponding group values CP+LH, Tukey HSD test, p<0.05

tion of LP/AOS markers was performed photometrically on a semi-automatic biochemical analyzer "MapLab plus" (BSI, Italy) and spectrophotometer SF-46 (LOMO, Russia).

Statistical Analysis

Descriptive statistics methods were used for preliminary statistical processing of results. The results were expressed as the arithmetic mean (M) and the standard error of the mean (SEM). For further comparison of samples, parametric a priori (one-way analysis of variance ANOVA) and a posteriori (Tukey HSD test) methods of analysis were used. Differences were considered significant in terms of significance level p<0.05. Statistical processing was performed using the basic software package MS Excel 2007 and IBM SPSS Statistics 22.[28]

Results and Discussion

Daily irrigation of 0.05 ml of atropine sulfate to the oral surface for 14 days significantly reduced the rate of spontaneous salivation and total volume of saliva (Table 2).

After irrigating the oral cavity of rats with individual solutions of AsA (group CP+AsA) and LH (group CP+LH), no effect was observed on the pathologically reduced parameter of salivation rate in animals on the background of experimental xerostomia. In addition, in animals treated with oral AsA solution, there was a slight decrease in salivary pH compared with intact control (IC), which can be explained by the acidic nature of this API.[15]

In the case of therapeutic and prophylactic use of the combined composition solution of AsA and LH (group CP+AsA+LH) there was a significant increase in salivation during the observation period – 2.17 times compared to the same value in the group of control pathology (CP). At the same time statistical differences from normal value of IC were not noticed. The result proves the summation of effects of the active ingredients, which is indicated by the significant differences from both comparison groups. It should be noted that the pH of saliva did not change compared to IC. The results are given in Table 2.

Daily oral cavity irrigation with atropine sulfate in experimental animals caused a statistically significant increase in the mass coefficients of the parotid and submandibular salivary glands (Table 3). According to the literature, this effect is due to the nar-

Table 4: LP/AOS markers in the homogenate of the submandibular salivary gland of rats with experimental xerostomia

Experimental group	TBA-reactants, μmol / g	RG, μmol / g	Catalase, μmol / (min*l)/g
IC	15.43 ± 0.76	2.69 ± 0.16	34.94 ± 2.61
CP	35.16 ± 3.74*	1.75 ± 0.11*	21.78 ± 1.40*
CP+AsA	26.10 ± 1.30*/**	2.16 ± 0.13*	23.54 ± 1.10*
CP+LH	19.78 ± 1.10**	1.95 ± 0.11*	26.89 ± 1.53*
CP+AsA+LH	17.01 ± 1.10**/#	2.36 ± 0.13**	32.89 ± 1.36**/#

** – differences are significant relative to the corresponding group values IC, Tukey HSD test, p<0.05*

*** – differences are significant relative to the corresponding group values CP, Tukey HSD test, p<0.05*

 # – differences are significant relative to the corresponding group values CP+AsA, Tukey HSD test, p<0.05

– differences are significant relative to the corresponding group values CP+LH, Tukey HSD test, p<0.05

rowing of the salivary ducts, accumulation of saliva and development of a local inflammatory process in the salivary glands.[22-24,29] It should be noted that the mean value of the sublingual salivary mass was also slightly increased, but the differences between the two controls were not significant.

Irrigation with AsA or LH solutions separately in the studied regime did not lead to any changes, and the average values of mass coefficients of all removed salivary glands in these groups did not differ statistically from similar indicators of the CP group.

The use of the composition of AsA and LH contributed to a significant decrease in the mass coefficient of the parotid salivary glands in comparison with this indicator in the CP group (13.0%; p<0.05), but no significant differences from similar indicators were observed in both reference groups.

The mass coefficient of the submandibular salivary gland under the action of the combination of AsA and LH has normalized and was statistically lower than similar in the CP group (by 18.1%; p<0.05) and both comparison groups (by 15.4%, p<0.05 vs CP+AsA, by 13.6%, p<0.05 vs CP+LH), which also indicates the potentiation and summation of the effects of the active ingredients composition. The results are presented in Table 3.

In the homogenate of submandibular salivary gland of rats with experimental xerostomia, a significant imbalance of markers of pro-/antioxidant system was observed. Thus, during the experiment there was a significant increase in the content of thiobarbituric acid reactants (TBA-reactants), a decrease in the content of reduced glutathione (RG) and inhibition of catalase activity, which indicates an increase in free radical processes in the affected tissues (Table 4).

Thus, during the use of a solution of AsA in the homogenate of the salivary gland the content of TBA-reactants was probably decreased by 25.8% compared with this indicator in the group of CP. At the same time, when using the solution of LH, the same indicator was decreased by 43.7% (p<0.05 vs CP). In this case, no significant effect on the content of RG or on the activity of catalase in any of the comparison groups was observed.

In contrast to monotherapy with individual components using a solution of the composition of AsA and LH, normalization of the antioxidant status of the studied tissue up to almost intact level was registered. Thus, in this group, the level of TBA-reactants in the tissue homogenate probably decreased by 51.6%, RG content probably increased by 34.9%,

and catalase activity probably increased by 51.0% compared to similar indicators from the CP group. The results are presented in Table 4.

Conclusions

Against the background of irrigation of the oral cavity of rats with experimental anthropine-induced xerostomia with a combined solution of the composition of lysozyme hydrochloride and ascorbic acid at a dose of 4 and 8 mg/kg, respectively, in 14 days the corrected rate of spontaneous salivation glands and the antioxidant status of the tissues of the submandibular salivary gland to the level of intact animals was observed. In addition, the use of the test composition did not lead to changes in the pH of saliva, which is an additional factor of the good safety profile.

Given that monocomponent therapy with each of the active ingredients provides no significant effect on the studied indicators, on the contrary, their use in combination stipulates modification and enhancement of the therapeutic effect.

The presence of a significant corrective effect on the studied indicators determines the expediency of development and further study of dental medicated chewing gums with a combination of lysozyme hydrochloride and ascorbic acid for symptomatic treatment of patients with xerostomia. □

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ΦΑΡΜΑΚΕΥΤΙΚΗ, 33, IV, 2021 | 304-311 ΕΡΕΥΝΗΤΙΚΗ ΕΡΓΑΣΙΑ

PHARMAKEFTIKI, 33, IV, 2021 | 304-311 RESEARCH ARTICLE

Development and Validation of a Titrimetric Method for Quantitative Determination of Free Organic Acids in Green Tea Leaves

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KEYWORDS:
green tea leaves; organic acids; titrimetric method; validation

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ABSTRACT

Introduction. Organic acids are a groups of compounds exhibiting a wide range of biological activities and some of them are included in different medicines. The search for new plant sources rich in organic acids, as well as the development of methods for their quantitative determination in plant raw materials is an interesting topic of research.

The aim of study was to develop and validate a titrimetric method with potentiometric detection of the end-point for quantitative determination of free organic acids in green tea leaves.

Research methods. A titrimetric method for the quantitative determination of free organic acids was developed and validated as per ICH guidelines.

Results and discussions. The linear regression data for the calibration curve showed a good linear relationship over the concentration range 0.72 % to 3.75%. Linear regression was found to be y = 0.2834x + 0.0164 (r = 0.9992). The percentage recovery was found to be in the range from 98.08 to 101.92 % with a %RSD value of 1.73 %. The %RSD of repeatability and intermediate precision were to be 1.76 and 1.59 %, respectively. The %RSD of stability was found to be 1.24 %. The method was confirmed to be accurate and precise having %RSD values of less than 2% in all studies. The amount of total free organic acids in green tea leaves was quantified to be 1.82 %.

Conclusions. The titrimetric method of quantitative determination of free organic acids in green tea leaves was developed and validated according to the following parameters: specificity, linearity, accuracy, repeatability, intermediate precision, stability. The proposed titrimetric method can be used for routine analysis for determination free organic acids in green tea leaves and quality control purpose as the developed method is simple, rapid, accurate and sufficiently precise.

Introduction

Organic acids are aliphatic or aromatic compounds, which are characterized by the presence of one or more carboxyl groups[1]. They are important in biological processes, since they are involved in various fundamental pathways in plant and animal metabolism and catabolism as intermediate or final products, playing a key role in the citric acid cycle or Krebs cycle[2].

Organic acids possess antioxidant, anti-inflammatory, antimicrobial and immunomodulatory activity. In addition, they create favorable conditions for the vital activity of beneficial intestinal microorganisms[3-6].

Green tea has a global popularity and includes several varieties of tea. The main compounds of chemical composition of green tea are phenolic acids, polyphenolic compounds, caffeine as well as amino acids, organic acids and fats[7]. Literature survey shows that green tea contains oxalic acid (0.3 – 1.5%), quinic acid (0.07 – 1%), citric acid (0.20%), succinic acid (0.25%) and malic acid (0.25%). Among the organic acids detected in tea, oxalic acid is of importance, as it might contribute to kidney stone formation[8]. However, in human studies, it has been shown that oxalic acid from tea did not dramatically elevate urine oxalic acid levels[9]. Other studies recognized a protection against urinary stones by tea due to its antioxidant properties[10].

A search of recent publications in Pubmed and ScienceDirect shows that there is no titrimetric method for quantitative determination of free organic acids in green tea leaves. In the contemporary literature, organic acids are determined by ion chromatography, capillary electrophoresis, gas chromatography and chromatography mass-spectrometry[11-16]. Chromatographic methods of analysis have been proposed for quantification of organic acids, as well. There is no doubt that chromatographic methods are precise and accurate, but they require qualified analysts, and relatively expensive instrumentation.

Therefore, a titrimetric method was chosen for the determination of organic acids in green tea leaves and the aim of the current study was to develop and validate such a method.

Materials and methods

The object of the study was green tea leaves (*Camellia sinensis L.*), which was collected in Anhui province, China. A Hanna 2550 pH meter with HI 1131P potentiometric electrode was used. All titrations were carried out manually. Free organic acids were titrated using a micro burette with Class A accuracy.

Citric acid was purchased from Sigma Aldrich (≥ 98 %), NaOH was of analytical grade. In order to prepare NaOH solution with the concentration of 0.05 M, 1.0 g of NaOH was dissolved in distilled water. The solution was diluted to 250 mL with the same water and standardized.

Preparation of total free organic acids from green tea

Dried leaves of green tea were grinded in the size 1-2 mm. The extraction of free organic acids was carried out by distilled water on water bath in a flask with a ground glass joint, which was equipped by a condenser and extracted for different time (from 30 to 120 min) at different temperature (from 20 º to 100 ºC), while the ratio of material to liquid ranged from 1:20 to 1:90 and extraction cycles ranged from 1 to 4.

Procedure for the quantitative determination of free organic acids

The content of free organic acids in the solution was calculated from the value of the equivalent volume of titrant. The equivalent volume of the titrant was determined by a constructed differential curve in the coordinates ΔE/ΔV - V. The equivalence point was fixed at the maximum on the constructed differential curve. The perpendicular line was dropped to the horizontal axis (Volume of the titrant) through the maximum and the volume of the titrant which was spent on titration was found (**Fig 1.**).

A blank experiment was also performed. According to it, the blank volume of 0.05 M sodium hydroxide was 0.03 mL.

The content of free organic acids (X, %) in terms of citric acid in completely dry raw materials was calculated by the following formula:

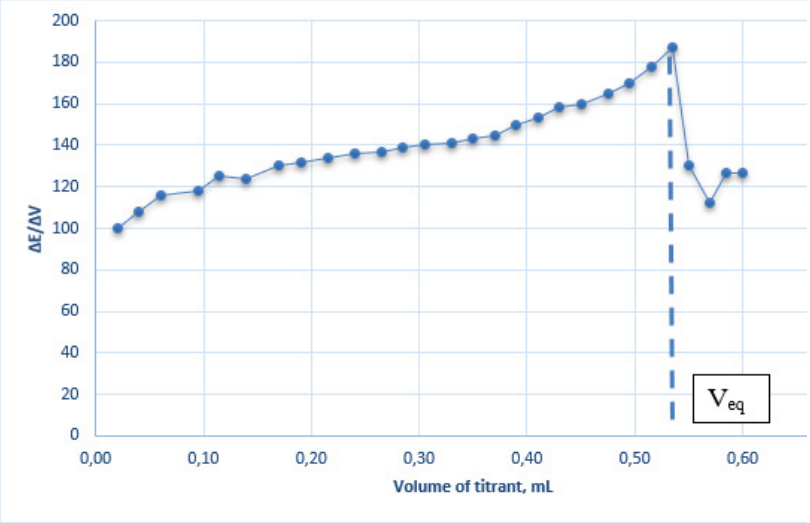

Figure 1. *Potentiometric titration curve of the determination of total free organic acids in green tea leaves*

$$X = \frac{(V_{eq} - V_x) \cdot 0.0032 \cdot 100 \cdot 100 \cdot 100 \cdot K}{m \cdot 5 \cdot (100 - W)}$$

where, 0.0032 – the amount of citric acid, which is equivalent to 1 mL of sodium hydroxide solution (0.05 mol/L), g; Veq is the volume of sodium hydroxide solution (0.05 mol/L), which was used for titration, mL; Vx – the volume of sodium hydroxide solution (0.05 mol/L), which was spent for titration in a blank experiment, mL; m –the mass of the raw materials used, g; K is correction coefficient for 0.05 mol/L sodium hydroxide solution; W – the loss in mass upon drying of the raw materials, %.

Optimization of organic acids extraction

In order to find the best conditions for the extraction of organic acids, the effects of the following influencing factors on extraction were evaluated: ratio of material to liquid, temperature, extraction cycles and duration of extraction. Factors are displayed in **Table 1**.

Validation

Validation of the titrimetric method for the quantitative determination of the amount of free organic acids in green tea leaves by potentiometric titration was performed according to the International Conference on Harmonization (ICH) guidelines[18]. The titrimetric method proposed was validated on the following parameters: specificity, accuracy, linearity, repeatability, intermediate precision, stability.

The specificity of the method was studied by potentiometric titration of the solvent.

The accuracy was verified by the method of additives in a triplicate analysis of three levels of concentration of free organic acids corresponding to 40, 60, 80 % of the working concentrations of free organic acids. The standard solution of citric acid was prepared as follows: 0.072 g (accurate weight) of citric acid was placed in a 200.0 mL volumetric flask, and the solution was diluted to the volume with distilled water. Then, an aliquot of the resulting standard solution of 2.00, 3.00, 4.00 mL was taken and placed in a 100 mL flask. After that 5.00 mL of the extract obtained from green tea leaves was added to the 100 mL flask, then 45.0 mL of distilled water was added, and the solution was titrated. The evaluation criterion in determining the accuracy was the value of the relative standard deviation (RSD), which according to the requirements should be not more than 2 %, and the percentage of recovery should be from 95 to 105 %.

The linearity of the method was studied at 9 concentration levels (40, 60, 80, 100, 120, 140, 160, 180, 200%) of the theoretical content of the total amount of free organic acids (calculated with reference to citric

Table 1: The influence of various factors on the completeness of the extraction of free organic acids from green tea leaves

Ration of material to liquid	Temperature, ºC	Extraction cycles	Duration of extraction, min	The total content of free organic acids*, %±CI
ration of material to liquid				
1:20	100	1	30	1.48±0.07
1:30				1.32±0.11
1:50				1.18±0.06
1:90				1.05±0.10
temperature				
1:20	20	1	30	1.19±0.06
	40			1.24±0.13
	60			1.30±0.07
	80			1.40±0.22
	100			1.45±0.15
extraction cycles				
1:20	100	1	30	1.50±0.11
		2		0.32±0.04
		3		0.10±0.01
		4		0.03±0.01
duration of extraction				
1:20	100	2	30	1.57±0.06
			60	1.64±0.06
			90	1.75±0.10
			120	1.81±0.04

** Average of five measurements, CI - confidence interval*

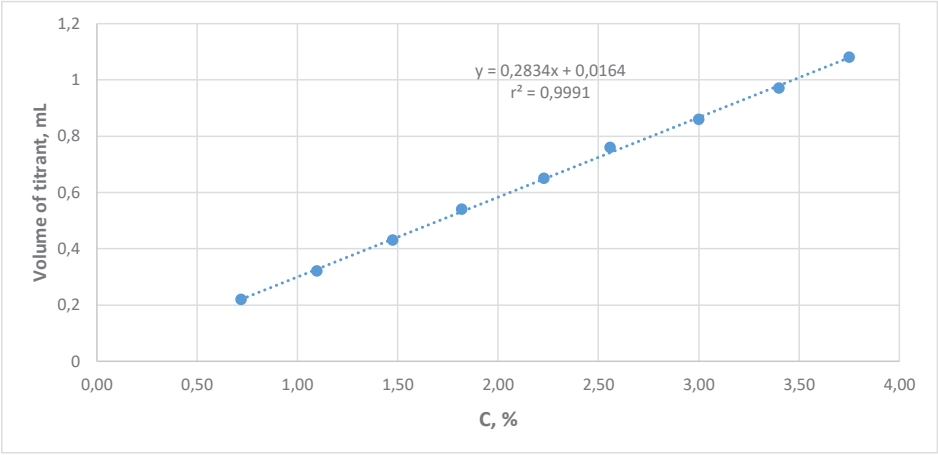

Figure 2: *Calibration curve of the titrant volume against concentration of free organic acids*

acid, %) in green tea leaves. In order to evaluate linearity of the method, different masses of raw material were taken 0.8; 1.2; 1.6; 1.8; 2.0; 2.4; 3.2; 3.6; 4.0 g (exact mass). After that each mass was extracted by the developed procedure. The quantitative content of the total amount of free organic acids in green tea leaves in the obtained solutions was then quantified by titrimetric method of titration. The linear regression was calculated by the method of least squares to obtain the regression equation and determine the correlation coefficient (r). According to the requirements of ICH, the value of the correlation coefficient when studying the linearity of the analytical method for determining the quantitative content of the active substance should be ≥ 0.999.

The repeatability of the method was checked by preparing an extract of green tea leaves from 6 portions of the raw material within a short period of time using the same set of reagents and with the participation of the same analyst. The intermediate precision was determined as described above in the same laboratory, but in different days. The acceptance criterion is expressed by the value of the %RSD, which should not exceed 2 %.

The stability of the potentiometric procedure was carried out, too. The acceptance criterion is expressed by the value of the relative standard deviation, which should not exceed 2 %.

Table 2: The results of the titration to prove the specificity of the developed method

$V_{titrant,}$ mL	Content of free organic acids, %	Statistical analysis*
Blank experiment (titration of distilled water)		
0.03	0.05	0.045±0.02% s_x = 0.0014
0.02	0.035	
0.03	0.05	
Results of titration of extract of green tea leaves		
0.57	1.00	1.00±0.02% s_x = 0.0033
0.56	0.99	
0.57	1.00	

**Average of three measurements, P = 95%*

The statistical processing of experimental data obtained was performed in accordance with the monograph «Statistical analysis of the results of a chemical experiment» of the State Pharmacopeia of Ukraine.

Table 3: Recovery studies by standard additions technique

Amount present, g	Amount added of citric acid, g	Amount taken of organic acids, g	Amount recovered, g	Recovery, %	RSD, %
0.036	0.015	0.052	0.051	98.08	1.73
			0.053	101.92	
			0.052	100.00	
0.036	0.022	0.058	0.059	101.72	
			0.058	100.00	
			0.057	98.28	
0.036	0.029	0.065	0.065	100.00	
			0.066	101.54	
			0.064	98.46	

Results and diccussions

It was found that the most complete extraction of organic acids from green tea leaves can be achieved by 2 cycles of extraction at 100 ºC with the ratio of material to liquid – 1:20 within 120 min. The results of study are represented in **Table 1**.

According to the obtained results, a procedure for the quantitative determination of free organic acids in green tea was developed: 2.0 g (exact mass) of the grinded raw material (2.0 mm) was placed in a 100 mL flask with ground glass joints, poured with 40 mL of distilled water, then the flask was equipped with a condenser and kept for 120 min in boiling water bath at 100 ºC. Extraction was repeated once more. After cooling, the solutions were quantitatively transferred into a 100 mL volumetric flask and make up to the mark by distilled water (solution A).

5.00 mL of the solution A was placed in a 100 mL flask and 45.0 mL of distilled water was added. After adding each portion of sodium hydroxide, the solution was mixed thoroughly and the electrode potential was recorded.

The method was validated according to the ICH

Table 4: Repeatability results for free organic acids in green tea leaves

Number of samples	Content of free organic acids, %
1	1.86
2	1.81
3	1.83
4	1.85
5	1.77
6	1.83
Mean, %	1.83
SD	0.0321
Confidence interval (P=95%), %	0.03
RSD, %	1.76

Table 5: Intermediate precision results for free organic acids in green tea leaves

Number of samples	Content of free organic acids, %	
	The first day	The second day
1	1.83	1.85
2	1.81	1.86
3	1.86	1.80
4	1.84	1.83
5	1.82	1.87
6	1.78	1.79
Mean, %	1.82	1.83
SD	0.0269	0.0311
Confidence interval (P=95%), %	0.02	0.02
Mean, %	1.48	1.69

Table 6: Stability result for free organic acids in green tea leaves

t, min	Content of free organic acids, %
0	1.85
15	1.82
30	1.81
45	1.80
60	1.79
Mean, %	1.81
SD	0.0225
Confidence interval (P=95%), %	0.02
RSD, %	1.24

guideline for the Validation of analytical procedures Q2((Q1A (R2) 2003, Q2A 1994, Q2B 1996). The extracts of green tea were prepared as per the procedure given above.

When studying the specificity of the method, it was shown that the solvent used in the samples preparation and the probable impurities did not affect the result of the quantification of the amount of free organic acids in green tea leaves (**Table 2**).

Linearity was proven in the concentration range from 0.72 % to 3.75%. The regression equation of the curve had the following form: y = 0.2834x + 0.0164. The value of the correlation coefficient (r) was equal to 0.9991 (**Fig. 2**).

The accuracy of the method was assessed using the percentage of recovery and the relative standard deviation. The percentage of recovery was found to be in the range from 98.08 to 101.92 %, the value of the %RSD when assessing the correctness of the method was 1.73 % and did not exceed 2 % (**Table 3**).

The precision of the method was confirmed by repeatability and intermediate precision. The values of %RSD for repeatability and intermediate precision were 1.76 and 1.59 %, respectively. The %RSD values were less than 2 % proving that the method is precise (**Tables 4,5**).

The stability of the method was determined for 60 min. It was found that the RSD value was 1.24 %. The %RSD value was less than 2 %, showing that analytical solution is stable during 60 min and there is no significant change in the result (**Table 6**).

Conclusions

The titrimetric method for quantitative determination of free organic acids in green tea leaves was developed and validated according by the following parameters: specificity, linearity, accuracy, repeatability, intermediate precision and stability. The proposed titrimetric method can be used for routine analysis for determination free organic acids in green tea leaves and quality control purpose as the developed method is simple, rapid, accurate and sufficiently precise. □

Conflict of Interests: *None declared.*

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