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Rodolfo Cabral Marcelino

Molecular docking study involving bioactive natural compounds against SARS-CoV-2 proteins

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Orientador: Prof. Dr. Nelson José Freitas da Silveira

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RODOLFO CABRAL MARCELINO

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“You must unlearn what you have learned.”

Master Yoda

RESUMO

No final de 2019, casos atípicos de pneumonia acompanhados de sintomas particulares começaram a ser relatados na cidade chinesa de Wuhan. Após algumas semanas, a doença acima mencionada se espalhou por toda a China e se tornou uma pandemia alguns meses depois. Síndrome Respiratória Aguda Grave Coronavírus 2 (SARS-CoV-2) é o agente etiológico dessa pneumonia atípica, que foi denominada Doença de Coronavírus 2019 (COVID-19). Em uma tentativa de encontrar uma terapia eficiente para combater a propagação da COVID-19 ou para curar pessoas infectadas a partir dos danos de seus tecidos, pesquisadores ao redor do mundo têm estudado várias plantas, ervas e produtos naturais. Estes medicamentos potenciais têm sido utilizados para fins terapêuticos ao longo dos anos devido à grande variedade de compostos bioativos que os caracteriza. O presente trabalho teve como objetivo realizar triagens virtuais utilizando o software AutoDock Vina para executar estudos de acoplamento molecular de estruturas tridimensionais (3D) de compostos naturais bioativos conhecidos contra 24 proteínas SARS-CoV-2. Analisamos escores de energia (fornecidos em -Kcal/mol), bem como interações intermoleculares entre proteína-ligante. Os ensaios *in vitro* e *in vivo* podem ser realizados para contribuir com nossas descobertas e, portanto, verificar se as substâncias químicas derivadas de plantas e ervas, tais como *Punica granatum*, *Centella asiatica* e *Solanum spp.*, como promissoras candidatas a drogas para tratar ou prevenir a COVID-19.

Palavras-chave: proteínas de SARS-CoV-2; Ancoragem Molecular; Compostos Naturais Bioativos.

ABSTRACT

At the end of 2019, atypical cases of pneumonia accompanied by particular symptoms started being reported in the Chinese city of Wuhan. After a few weeks had passed, the aforementioned disease spread throughout China and became a global pandemic some months later. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is the etiological agent of that atypical pneumonia, which has been named Coronavirus Disease 2019 (COVID-19). In an attempt to find an efficient therapy to either fight the spread of COVID-19 or to cure infected people from its tissue damages, researchers around the world have been studying several plants, herbs and natural products. These potential medicines have been used for therapeutic purposes throughout the years due to the great variety of bioactive compounds characterizing them. The present work aimed to perform virtual screenings by using AutoDock Vina software to execute molecular docking studies of tridimensional (3D) structures of well-known bioactive natural compounds against 24 SARS-CoV-2 proteins. We have analyzed energy scores (provided in -Kcal/mol) as well as ligand-protein intermolecular binding interactions. In vitro and in vivo assays should be furtherly conducted to corroborate with our findings and therefore validate chemical substances derived from plants and herbs, such as *Punica granatum, Centella asiatica* and *Solanum spp.*, as promising drug candidates to either treat or prevent COVID-19.

Keywords: SARS-CoV-2 proteins; Molecular docking; Bioactive natural compounds.

FIGURES LIST

POD = peroxidase, LDH = lactate dehydrogenase, Nrf2=, HO-1 = heme oxygenase-1, Bcl2 = B-cell lymphoma-2 apoptosis regulator, FSH = follicle stimulating hormone, LH = luteinizing hormone, T = testosterone. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).....56

TABLES LIST

LIST OF ABREVIATIONS

ACE2 - Human Angiotensin- Converting Enzyme 2

ADT - AutoDock Tools

ARG - Arginine

COVID-19 - Coronavirus Disease 2019

CXCL10 - IFN-γ-inducible protein 10

DNA - Deoxyribonucleic Acid

FDA - Food and Drug Administration

GUI - Graphical Interface

HCC hepatocellular carcinoma

HIS - Histidine

IL- Interleukin

Kcal/mol – Kilocalories / mol

MCP - Monocyte chemotactic protein

MeSH - Medical Subject Headings

MNP - Microplastics and Nanoplastics

Nsp - Nonstructural Proteins

ORF - Open reading frame

PDB - Protein Data Bank

PP - Plastic particles

PRO - Proline

PROSPERO- International Prospective Systematic Review Registry

RdRp - RNA-directed RNA polymerase

RMSD - Root-Mean Square Deviation

RNA - Ribonucleic acid

SARS-CoV-2 - Síndrome Respiratória Aguda Grave Coronavírus 2

SER - Serine

SSA - Saikosaponin A

SSB2 - Saikosaponin B2

SSD - Saikosaponin D

THR - Threonine

TNF-α - Tumour Necrosis Factor alpha

VAL - Valine

WHO - World Health Organization

SUMMARY

CHAPTER 1

Molecular docking study involving bioactive natural compounds against SARS-CoV-2 proteins.

Marcelino, Rodolfo Cabral, Leonardo Pereira de Araújo, João R. Bueno de Morais Borba, and Nelson José Freitas da Silveira. "Molecular docking study involving bioactive natural compounds against SARS-CoV-2 proteins". Natural Resources for Human Health (2022). doi:10.53365/nrfhh/147375.

ABSTRACT

At the end of 2019, atypical cases of pneumonia accompanied by particular symptoms started being reported in the Chinese city of Wuhan. After a few weeks had passed, the aforementioned disease spread throughout China and became a global pandemic some months later. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is the etiological agent of that atypical pneumonia, which has been named Coronavirus Disease 2019 (COVID-19). In an attempt to find an efficient therapy to either fight the spread of COVID-19 or to cure infected people from its tissue damages, researchers around the world have been studying several plants, herbs and natural products. These potential medicines have been used for therapeutic purposes throughout the years due to the great variety of bioactive compounds characterizing them. The present work aimed to perform virtual screenings by using AutoDock Vina software to execute molecular docking studies of tridimensional (3D) structures of well-known bioactive natural compounds against 24 SARS-CoV-2 proteins. We have analyzed energy scores (provided in -Kcal/mol) as well as ligand-protein intermolecular binding interactions. In vitro and in vivo assays should be furtherly conducted to corroborate with our findings and therefore validate chemical substances derived from plants and herbs, such as *Punica granatum, Centella asiatica and Solanum spp.*, as promising drug candidates to either treat or prevent COVID-19.

Keywords: SARS-CoV-2 proteins; Molecular docking; Bioactive natural compounds.

INTRODUCTION

In December of 2019, the world was struck by a new type of pneumonia with symptoms varying from fever to dry cough and dyspnea. The emergence of the virus causing this disease took place in Wuhan, China. Initially, that illness was named Wuhan pneumonia since the number of cases was restricted to the Wuhan region. However, due to the rapid virus spread and its associated disease's high infection rate in other countries, the World Health Organization (WHO) considered this pathogen a global threat and declared its resulting disease a pandemic on March 11, 2020. Through sequencing of the virus genome, it was possible to uncover that the causative agent of this newly discovered pneumonia belongs to the coronavirus family, which is knowingly capable of infecting humans (Liu et al., 2020).

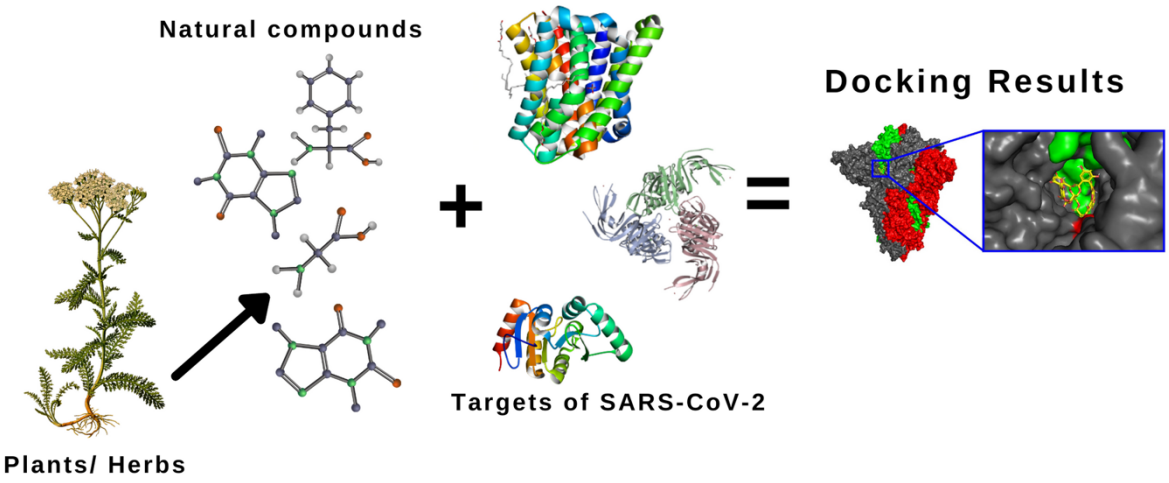
Coronaviruses are RNA-type microorganisms widely spread among humans, other mammals and birds, and they can cause respiratory, enteric and neurological disorders. The etiological agent of the aforementioned atypical pneumonia has been named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), and its consequent disease has been named Coronavirus Disease 2019 (COVID-19).

Initially, the SARS-CoV-2 spike glycoprotein binds to human angiotensin-converting enzyme 2 (ACE2) in order to infect cells. Then, its transcription machinery produces four structural proteins, namely envelope small membrane (E), spike (S), nucleocapsid (N) and membrane (M), but also a myriad of accessory proteins, such as open reading frame (ORF) 3a, 8 and 9b, as well as replicase polyproteins that are proteolytically cleaved into sixteen nonstructural proteins (Nsps), including papain-like protease/PLpro(Nsp3), 3C-like protease (Nsp5), Nsp9, RNA-directed RNA polymerase/RdRp (Nsp12) and helicase (Nsp13) (Liu et al., 2020).

After the pathophysiology of SARS-CoV-2 was discovered, an incessant search began for a drug or compound capable of fighting its replication and consequent infectious disease process. Different drugs and active ingredients have been tested at the same time that several countries have started a race to produce an effective vaccine against COVID-19 (Wu et al., 2020). Numerous steps are necessary to verify both the efficacy and the safety of potential new drugs when it comes to validating studies involving SARS-CoV-2. Techniques pertaining to areas of Molecular Biology, Bioinformatics, Immunology, Pharmacodynamics and Pharmacokinetics are

imperative for the development of compounds that can fight COVID-19 (Pan et al., 2013). In this context, molecular docking is a computational experiment that aims to search for the leading interactions between a target (receptor/protein) and a given ligand. This bioinformatic technique takes into account the best fit as well as the finest interaction comprising a target, e.g., protein binding sites, and the specific geometry of a ligand, so that an associated binding energy score can be generated. In this type of experiment, specific in silico techniques are used to analyze protein-ligand interactions as well as different ligand conformations that are obtained as a result of a complex formed with a protein's active or allosteric site (Azevedo & Walter, 2019).

Nowadays, one of the most widely used computer programs on the molecular docking realm is AutoDock Vina, a software developed by Trott and Olson (2010) and whose main application is to perform rigid-flexible molecular docking screenings. In this type of in silico methodology, the chosen target remains rigid (with no rotation, no translation and no torsion), while its associated ligand has enough flexibility to generate distinct conformations (positions), out of which a favorable interaction can be established (Trott & Olson, 2010).



GRAPHICAL ABSTRACT- Steps realized for execution of this study and docking

Medicinal plants have been utilized to combat a multitude of pathogens. This important fact may be due to the existence of various bioactive compounds found in natural plants (Figure 1). As a result, a great variety of drug classes originated from natural plants has emerged. Teas, poultices, decoctions and a myriad of other different forms of both extracting herbs' medicinal constituents and administering them have been used for centuries. Additionally, in the last decades, evaluations have been conducted to establish their promising applications on a therapeutic scope (Atanasov et al., 2015).

Natural compounds have a remarkable advantage over synthetic substances. Although both of these chemical entities have the same structure and therefore equal physicochemical and biological properties, compounds obtained from medicinal plants do not need to be synthetized. As a positive outcome, they do less harm to the environment since extraction techniques may use smaller quantities of organic solvents, which are, for the most part, knowingly toxic (Fuzimoto & Isidoro, 2020). For this reason, we analyzed several natural chemical compounds with the aim of verifying their efficacy against SARS-CoV-2 proteins through in silico investigations.

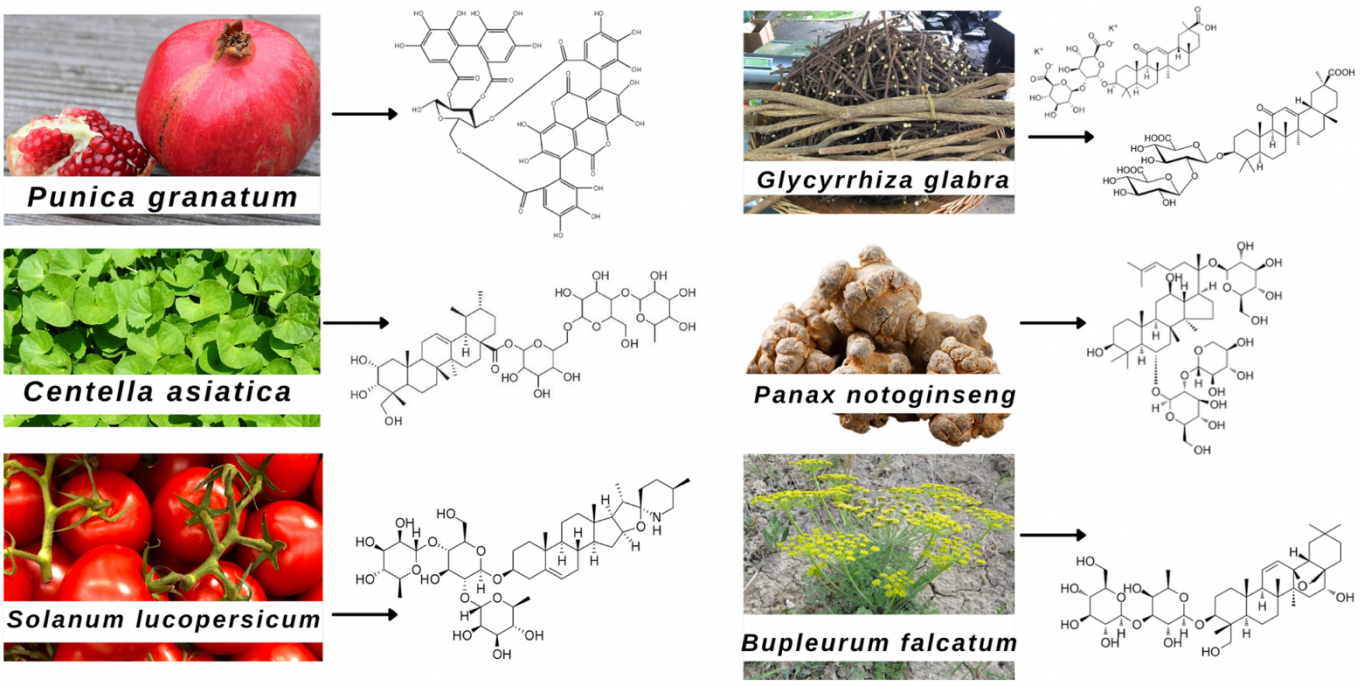


Figure 1. Plants and chemical structures of their bioactive natural compounds

MATERIAL AND METHODS

All natural compounds that comprise the Food and Drug Administration (FDA) library were chosen as ligands and then downloaded. Subsequently, all selected FDA structures were converted to *.pdbqt format using AutoDock Tools (ADT) v1.5.6, which was used as a graphical interface (GUI) to edit and generate all converted files.

With regard to the quest for potential targets in SARS-CoV- 2, the majority of proteins that we chose participate in the initial infection as well as in the subsequent viral replication process that characterizes COVID-19. A total of 24 proteins were chosen from PDB (Protein Data Bank) website (https://w ww.rcsb.org), and the most adequate files (the ones exhibiting the highest resolution) were downloaded in *.pdb format, which contains all information relating to the elected structures. Proteins were also prepared using ADT GUI. Each 3D protein structure file in *.pdb format was separately inserted and read into ADT GUI. Then, polar hydrogen atoms were added, and water molecules as well as other unwanted ligand molecules were removed so that there was no bias due to the presence of these chemical entities on the binding sites. Finally, all files were saved in *.pdbqt format.

Molecular docking assays were performed using AutoDock Vina v1.2.3. To ensure that ligands would interact with the regions where they exhibit the best fit in each of the 24 chosen proteins, molecular docking calculations were delimitated by a 3D space called grid box, in which Vina’s search algorithms test different positions with an eye to find the first-rate ligand conformation that complexes with the given target. The aforementioned grid box delimitations encompassed the whole proteins, for we wanted to extend our search to cover potential allosteric sites.

Results for all proteins and their respective complexed ligands were arranged in an increasing energy order (from most negative to most positive) for the purpose of determining the finest interaction profiles. Then, once the most favorable results had been obtained, the best ranked conformations of every ligand associated with each macromolecule were analyzed in order to dissect interaction profiles (Figure 2). In this context, we used PyMOL v2.5 as a molecular visualization system.

We analyzed all results from a scoring function perspective, also called score, which performs variation in rotational, translational, and conformational positions as

well as in motions to find the most stable target-ligand complex. Aside from that, hydrogen bonds (H-bonds) were also determined. To this end, we utilized Maestro v13.0. Finally, to validate our in silico results, we conducted redocking, and Root-Mean Square Deviation (RMSD) values were calculated using AutoDock Tools (ADT) v1.5.7 (Figure 3).

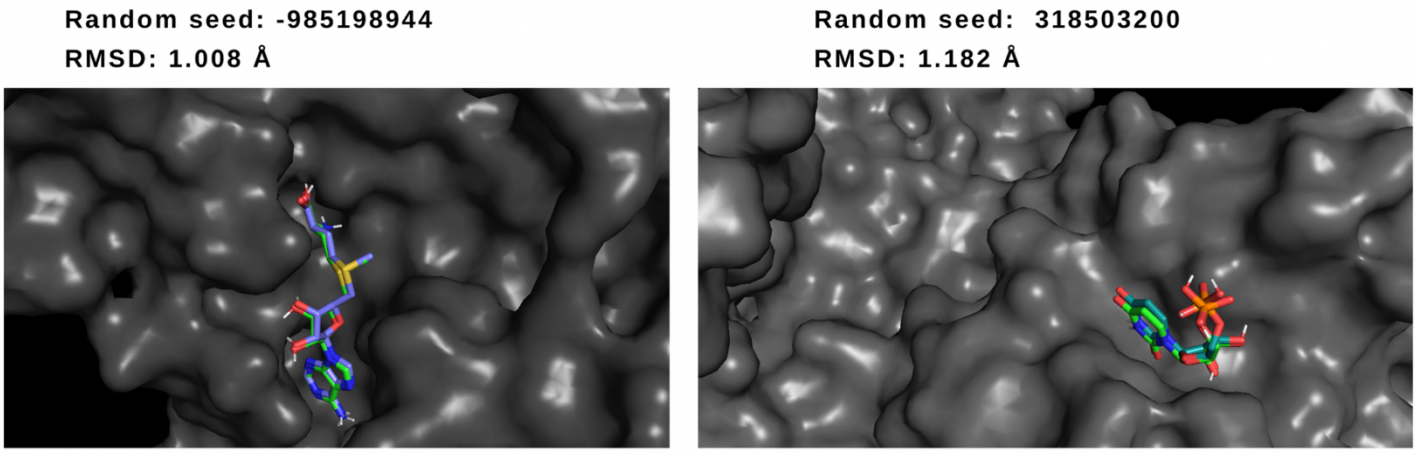


Figure 2. Visualization of 3D Molecules

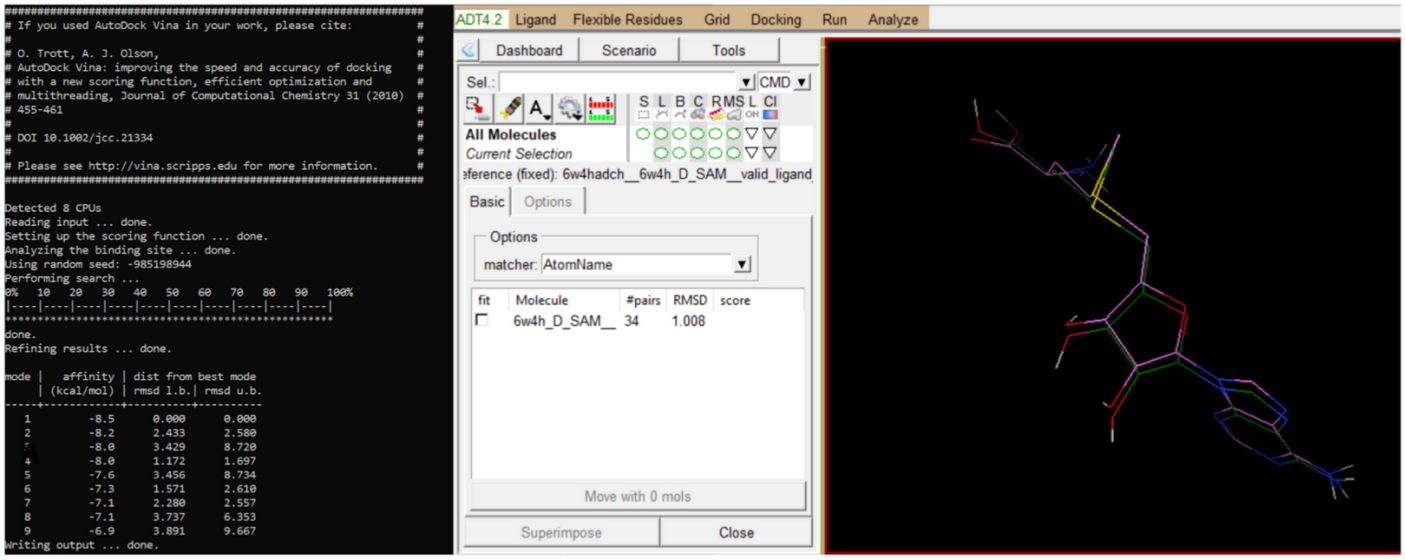


Figure 3. Docking Validation using AutoDock Vina

RESULTS AND DISCUSSION

In an attempt to find leading drugs and bioactive substances to either prevent or treat COVID-19, we noticed that green tea contains catechins and polyphenols, both of which may be effective in combating this disease. Additionally, these chemical entities are promising drug candidates capable of impairing viral replication, and their

activities against different types of viruses have inspired us to search for auspicious compounds capable of inhibiting SARS-CoV-2 initial infection and/or replication (Calland et al., 2012; Carneiro et al., 2016; Ismail & Jusoh, 2017).

Research studies show that several ligands are able to interact with various SARS-CoV-2 proteins, and their results exhibit a first-rate binding affinity between a given ligand and its associated protein (Senger et al., 2020). This type of binding energy between two molecules is based on the ”lock and key” scheme, a well-known strategy in computational drug design. The protein-ligand or even protein-protein perfect fit is mainly based on this concept. Specific interactions between two structures occur through H-bonds, van der Waals forces as well as other interacting bonds that result in a complex between two given molecules. In this context, researchers look for the lowest possible energy to generate stability, which takes place when two substances that form a complex come together, as previously described by the ”lock and key” scheme (G. Chen et al., 2020). Molecular docking screenings using AutoDock Vina v1.2.3 enabled us to find first-rate positions defined by binding energy scores between given ligands and their respective SARS- CoV-2 macromolecules (Table 1). Various bioactive substances, well-known for treating both neuroinflammation and neurodegenerative diseases, have demonstrated great interaction profiles with SARS-CoV- 2 proteins. Punicalagin is a natural compound present in pomegranate (*Punica granatum*) and consumed worldwide. This fruit is used in popular medicine because it helps treat chronic diseases, such as diabetes, atherosclerosis and inflammatory processes (Akhtar et al., 2015; Banihani et al., 2013; Danesi & Ferguson, 2017; Igdoura et al., 2013). The majority of bioactive compounds found in pomegranate, namely phytobiotics, flavonoids and anthocyanins, are from its peel. These substances possess therapeutic properties, including anti-inflammatory, antioxidant, hypoglycemic and antimicrobial effects (Grabež et al., 2020; Lansky & Newman, 2007; Saeed et al., 2018; Xu et al., 2022).

As showed in Table 2, punicalagin interacted with several macromolecules, mostly proteins related to SARS-CoV-2 replication process, such as RdRp-nsp7-nsp8 complex (7C2K, 7BV1 and 7BV2), 3CLpro, also named main protease – Mpro (7C2Q and 6LU7, in both immature and mature states, respectively), PLpro (6WX4), Nsp-16-Nsp10 complex proteins (6W4H and 6WKS), ORF3a protein (6XDC), Nsp7-Nsp8 replicase complex (6XIP) and Nsp-1 protein (7K3N). In addition, punicalagin also

interacted with spike glycoproteins (6LXT and 6ZGE). The best energy score results are the ones from the punicalagin-RdRp-nsp7-nsp8 complex. The interaction profile between punicalagin and 6ZGE is characterized by four H-bonds established with amino acid residues PRO 2222, HIS 2232, SER 3115 and SER 3123, all of which are disclosed in the scheme that represents (Figure 4).

Table 1
General characteristics of all SARS-CoV-2 proteins selected for molecular docking studies.

Authors	Country of origin	PDB code	DOI	Expression system
Shi et al. (2020)	China	7C01	https://doi.org/10.1038/s41586-020-2381-y	*Homo sapiens*
Xia et al. (2020)	China	6LXT	https://doi.org/10.1038/s41422-020-0305-x	*Escherichia coli*
Benton et al. (2020)	United Kingdom	6ZGE	https://doi.org/10.1038/s41586-020-2772-0	*Homo sapiens*
Mandala et al. (2020)	USA	7K3G	https://doi.org/10.1038/s41594-020-00536-8	*Escherichia coli*
Zinzula et al. (2021)	German	6ZCO	https://dx.doi.org/10.1016%2Fj.bbrc.2020.09.131	*Escherichia coli*
Semper et al. (2021)	Canada	7K3N	https://dx.doi.org/10.1016%2Fj.isci.2020.101903	*Escherichia coli*
Frick et al. (n.d.)	USA	6WEY	https://dx.doi.org/10.1021/acs.biochem.0c00309	*Escherichia coli*
Shin et al. (2020)	German	6W9C	https://doi.org/10.1038/s41586-020-2601-5	*Escherichia coli*
Jin et al. (2020)	China	6LU7	https://doi.org/10.1038/s41586-020-2223-y	*Escherichia coli*
Yin et al. (2020)	China	7BV1, 7BV2	https://doi.org/10.1126/science.abc1560	*Spodoptera frugiperda*
Littler et al. (2020)	Australia	6WXD	https://doi.org/10.1016/j.isci.2020.101258	*Escherichia coli*
Krafcikova et al. (2020)	Czech Republic	6W4H	https://doi.org/10.1038/s41467-020-17495-9	*Escherichia coli*
Kandwal and Fayne (2020)	Ireland	6WLC	https://doi.org/10.1080/07391102.2020.1825232	*Escherichia coli*
Kern et al. (2021)	USA	6XDC	https://dx.doi.org/10.1101%2F2020.06.17.156554	*Spodoptera frugiperda*
Flower et al. (2021)	USA	7JTL	https://dx.doi.org/10.1101%2F2020.08.27.270637	*Escherichia coli*

Table 2
Descriptions of all SARS-CoV-2 proteins selected for molecular docking studies.

Protein name	**PDB code**	**Resolution (Å)**	**Characterization Method**
Helicase/Nsp13	5RL9	1.79	1
$3CL^{pro}/M^{pro}$/Nsp5	6LU7	2.16	2
Spike glycoprotein (S2 subunit)	6LXT	2.90	2
Spike-ACE2 complex	6M0J	2.45	2
Nsp16-Nsp10 complex	6W4H	1.80	2
PL^{pro}/Nsp3	6W9C	2.70	2
PL^{pro}/Nsp3 (macrodomain)	6WEY	0.95	2
Nsp16-Nsp10 complex	6WKS	1.80	2
Nsp15	6WLC	1.82	2
PL^{pro}/Nsp3	6WX4	1.66	2
Nsp9	6WXD	2.00	2
ORF3a	6XDC	2.90	3
Nsp7-Nsp8 replicase	6XIP	1.50	2
ORF9b	6Z4U	1.95	2
Nucleoprotein	6ZCO	1.36	2
Spike glycoprotein	6ZGE	2.60	3
RdRp-nsp7-nsp8	7BV1	2.80	3
RdRp-nsp7-nsp8	7BV2	2.50	3
Spike glycoprotein (S1 subunit)	7C01	2.88	2
RdRp-nsp7-nsp8	7C2K	2.93	3
$3CL^{pro}/M^{pro}$/Nsp5	7C2Q	1.93	2
ORF8	7JTL	2.04	2
Envelope small membrane	7K3G	NA	4
Nsp1	7K3N	1.65	2

1 – X-ray Crystallography; 2 – X-Ray Diffraction (XRD); 3 – Electron Microscopy (EM); 4 – Nuclear Magnetic Resonance (NMR); NA – not applicable.

Table 3
Molecular Docking Results of Bioactive Natural Compounds against SARS-CoV-2 proteins.

PDB code	**Compounds**	**Energy ΔG - Kcal/mol**
7C2K	Punicalagin	-12.7
7BV2	Punicalagin	-12.1
6ZGE	Punicalagin	-11.7
5RL9	Glycyrrhizinate Dipotassium	-10.7
6ZCO	Ammonium Glycyrrhizinate	-10.5
6LXT	Punicalagin	-10.2
7BV1	Punicalagin	-10.1
6W9C	Saikosaponin D	-9.7
6W4H	Punicalagin	-9.6
7C2Q	Punicalagin	-9.5
6LU7	Punicalagin	-9.5
7K3G	Saikosaponin B2	-8.9
6XIP	Punicalagin	-8.9
6LGZ	Solamargine	-8.8
6WX4	Punicalagin	-8.7
6WEY	Solamargine	-8.6
6WKS	Punicalagin	-8.2
6XDC	Punicalagin	-8.2
6Z4U	Saikosaponin A	-8.2
6M0J	Solamargine	-7.9
7C01	Asiaticoside	-7.4
7K3N	Punicalagin	-7.0
6WXD	Notoginsenoside R1	-6.1
7JTL	-	-

Solamargine is a substance found in tomatoes (*Solanum lycopersicum*), potatoes (*Solanum tuberosum*), aubergine/eggplant (*Solanum melongena*) as well as in other plants belonging to *Solanum* genus species. That natural compound was described by Tang et al. (2022) as an anticancer substance that can effectively treat various types of malignant tumors, such as hepatocellular carcinoma (HCC). Its alpha form is a knowingly bioactive alkaloid found in *Solanum surattense*, which previously showed great binding affinity with SARS- CoV-2 Mpro (Hasan et al., 2020). Our results, on the other hand, exhibit that alpha-solamargine interacted through better binding energy scores with a spike-ACE2 complex (6M0J) as well as with PLpro macrodomain (6WEY). H-bond interactions between solamargine and 6M0J are shown on Figure 5. Licorice (*Glycyrrhiza glabra*) is a plant whose roots' chemical substances have been used as antioxidants to prevent cancer as well as natural compounds to inhibit the growth of Leishmania major promastigotes and amastigotes in infected mice.

Additionally, these chemical entities have been utilized to treat bacterial, fungal and viral infections (Chrzanowski et al., 2020; Sheikhi et al., 2022). In 2005, Hoever and collaborators discovered through molecular docking studies that glycyrrhizinate dipotassium and ammonium glycyrrhizinate, bioactive molecules found in *Glycyrrhiza glabra*, could increase antiviral activity of glycyrrhizin derivatives against SARS- CoV (Hoever et al., 2005). In 2020, Chrzanowski et al. (2020) disclosed that the aforementioned licorice chemical compounds enhanced glycyrrhizin potential binding to ACE2 (Chrzanowski et al., 2020). Our results show a probable multitarget function of licorice as glycyrrhizinate dipotassium and ammonium glycyrrhizinate interacted through extremely favorable binding energy scores of -10.7 kcal/mol and -10.5 kcal/mol with SARS- CoV-2 helicase/Nsp13 (5RL9) and nucleoprotein (6ZCO), respectively. In regard to 5RL9, glycyrrhizinate dipotassium formed three H-bonds with amino acid residues VAL 752, THR 770 and THR 974. All binding interactions established between glycyrrhizinate dipotassium and 5RL9 as well as between ammonium glycyrrhizinate and 6ZCO can be seen in detail on Figure 6 and 7, respectively.

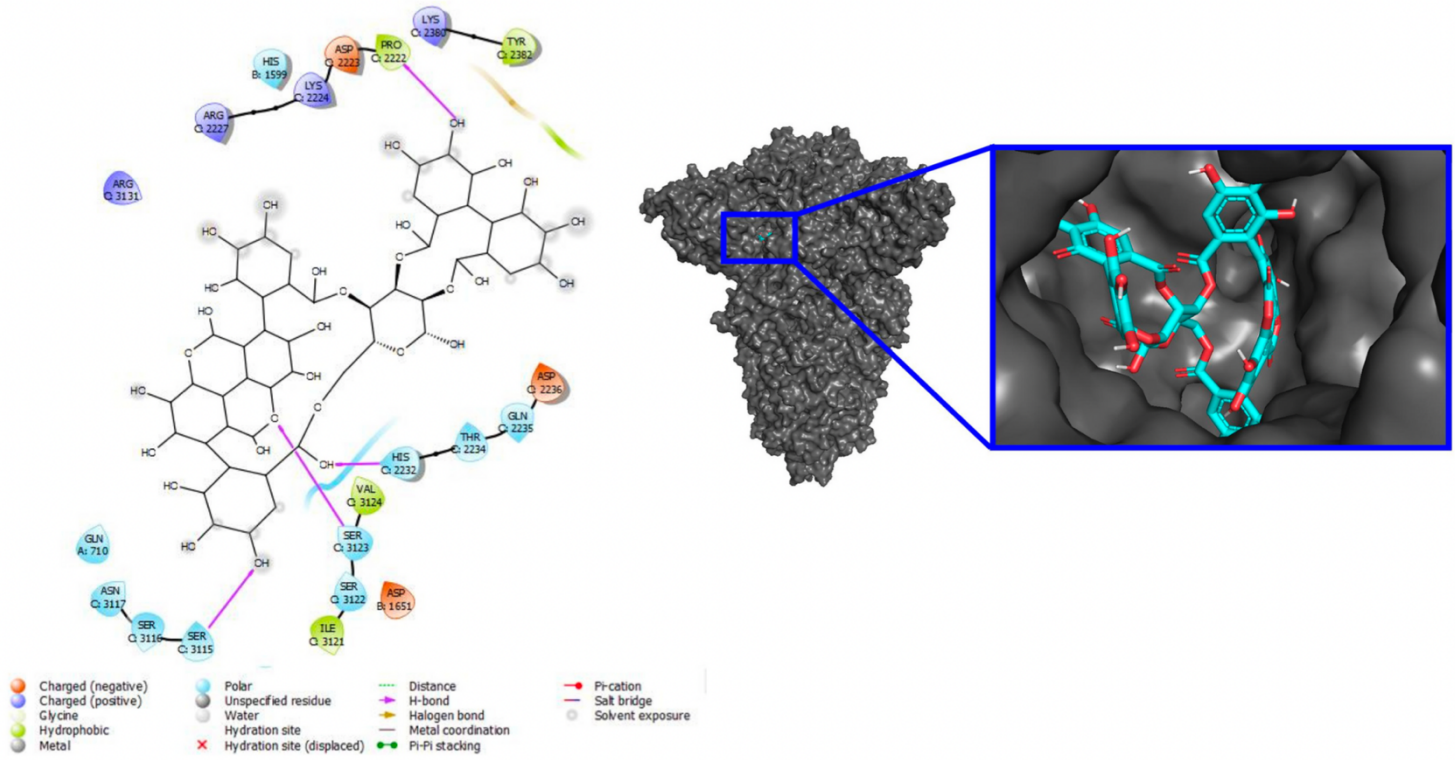


Figure 4. Interactions between Punicalagin and SARS-CoV-2 spike glycoprotein (6ZGE)

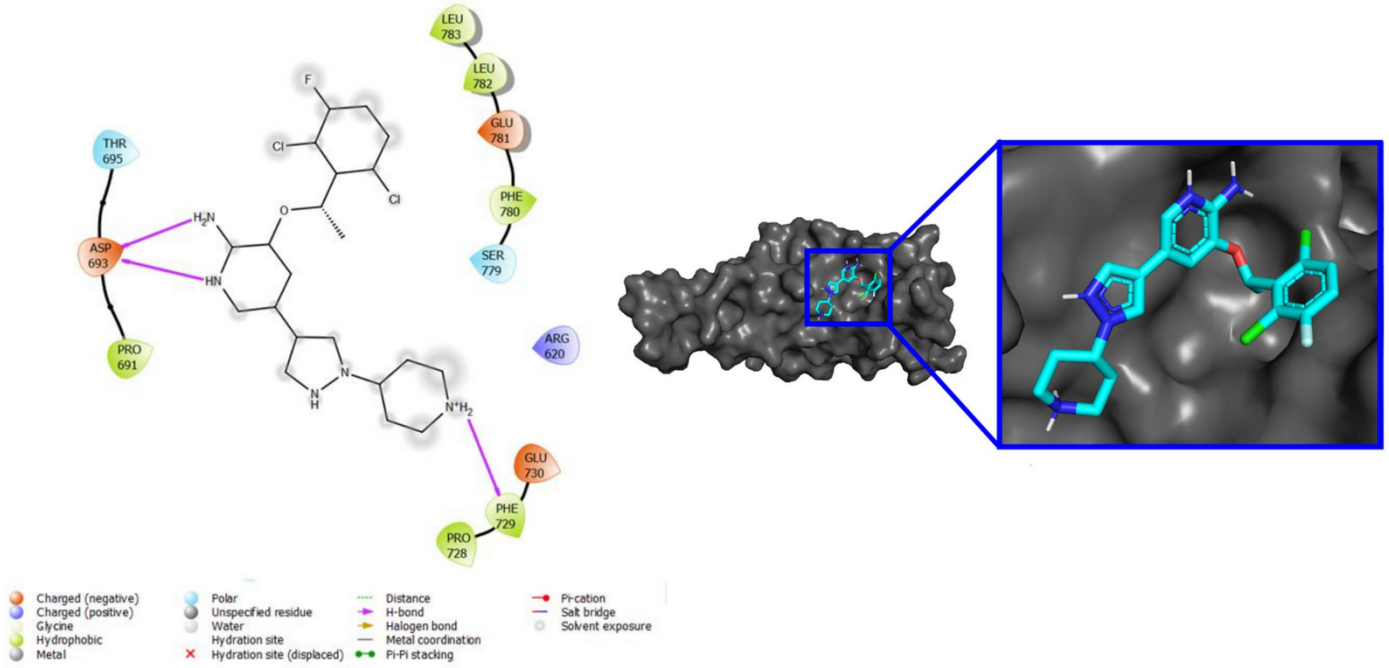


Figure 5. Interactions between Solamargine and spike-ACE2 complex (6M0J)

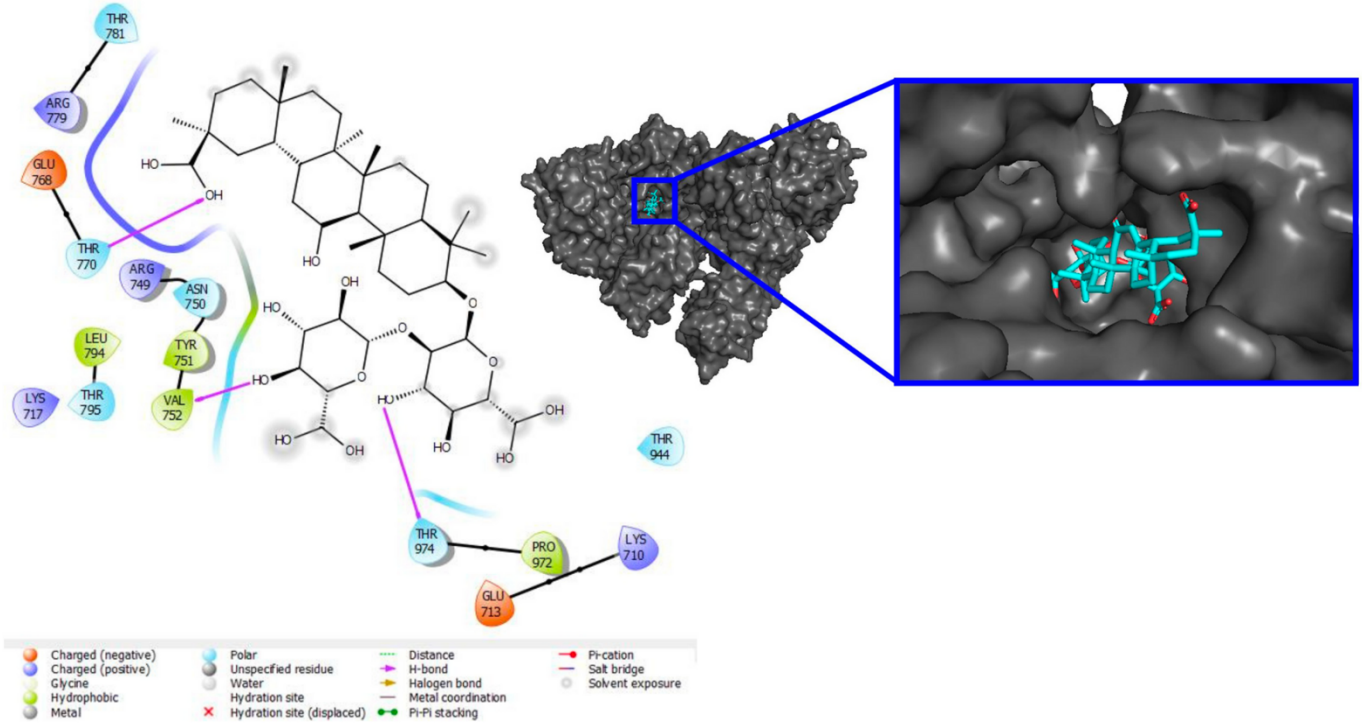


Figure 6. Interactions between Glycyrrhizinate Dipotassium and SARS-CoV-2 helicase (5RL9)

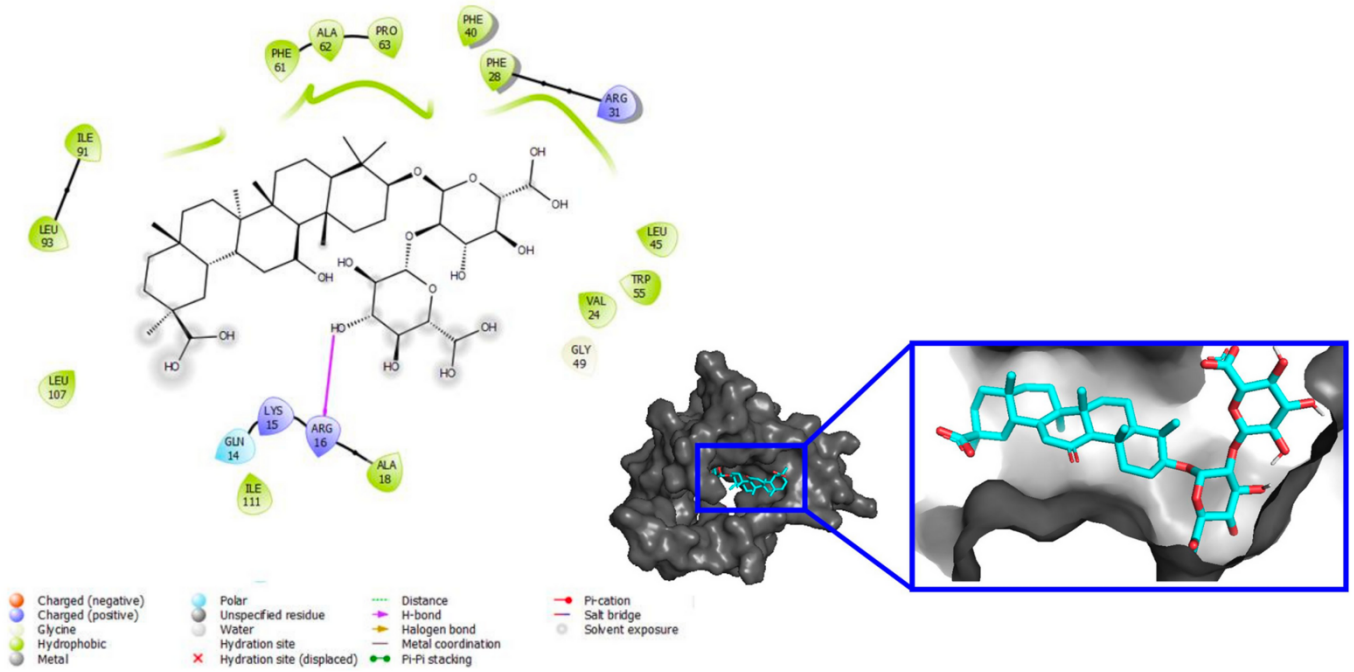


Figure 7. Interactions between Ammonium Glycyrrhizinate and SARS-CoV-2 nucleoprotein (6ZCO)

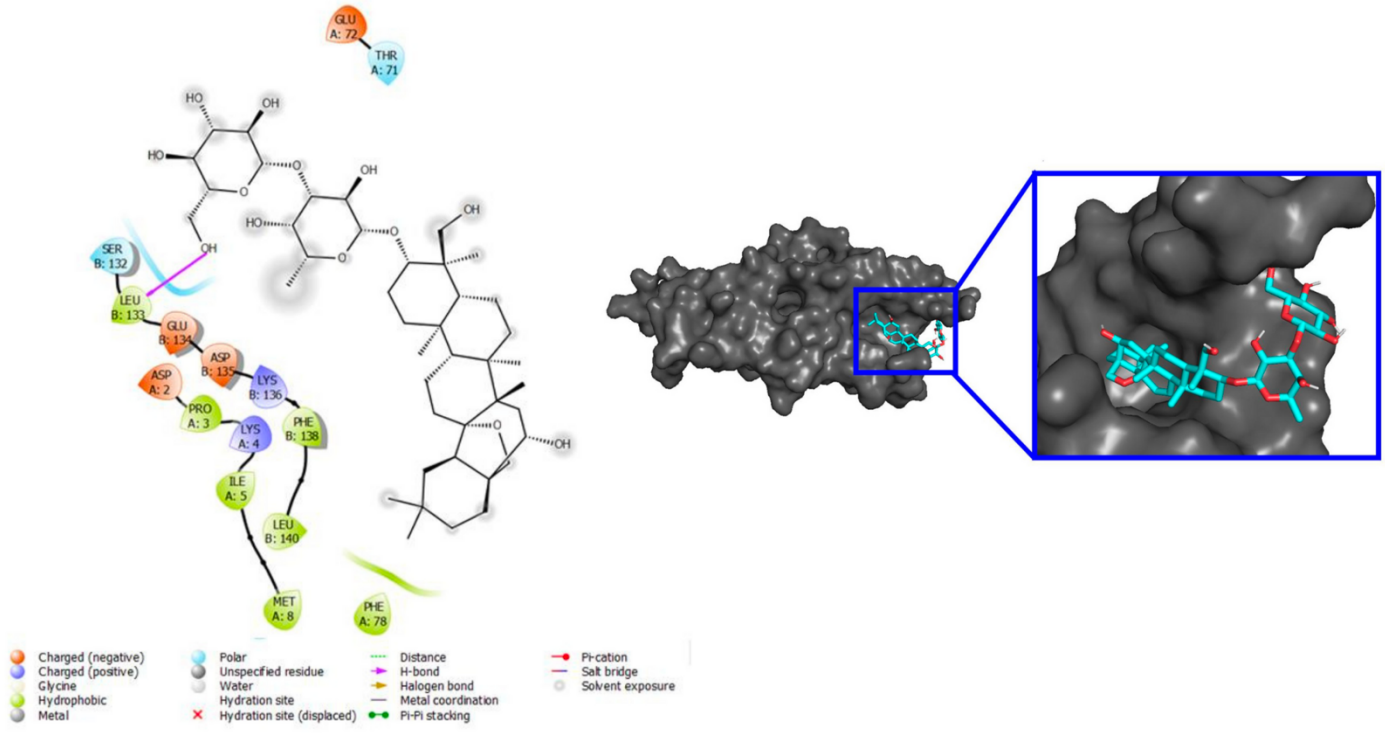


Figure 8. Interactions between Saikosaponin A and SARS-CoV-2 ORF9b protein (6Z4U)

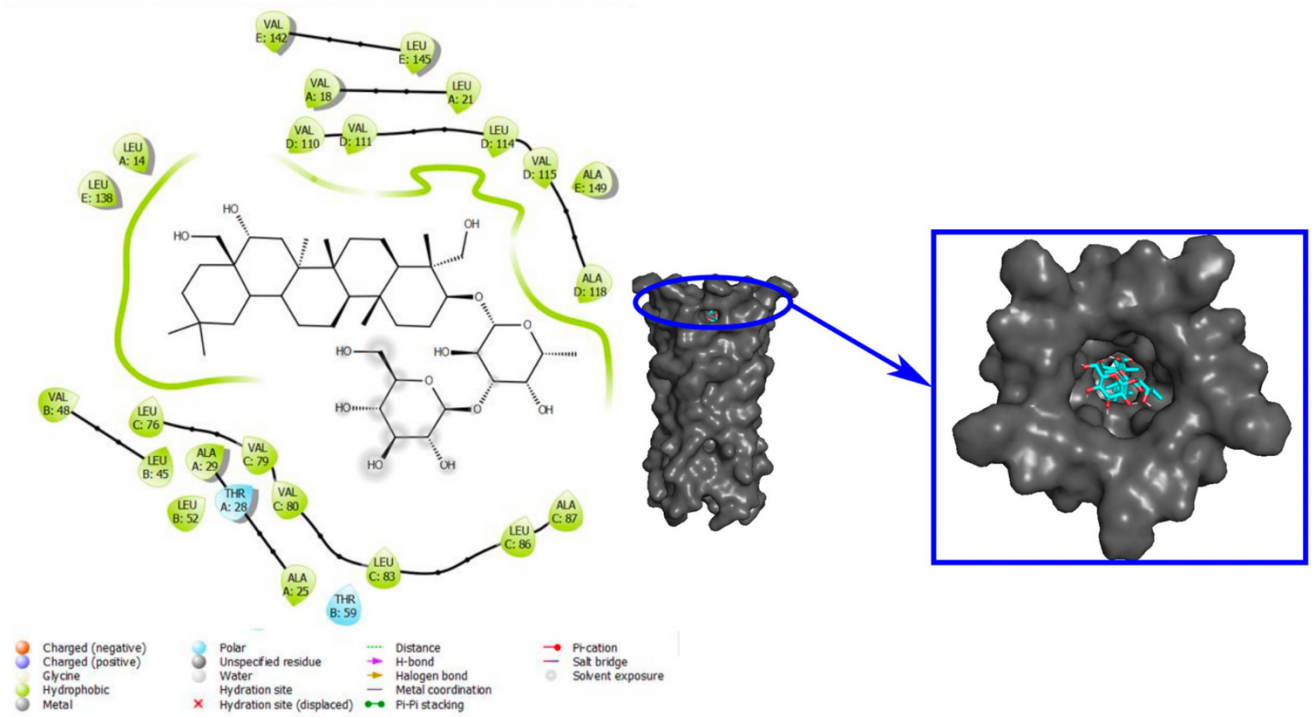


Figure 9. Interactions between Saikosaponin B2 and SARS-CoV-2 envelope small membrane protein (7K3G)

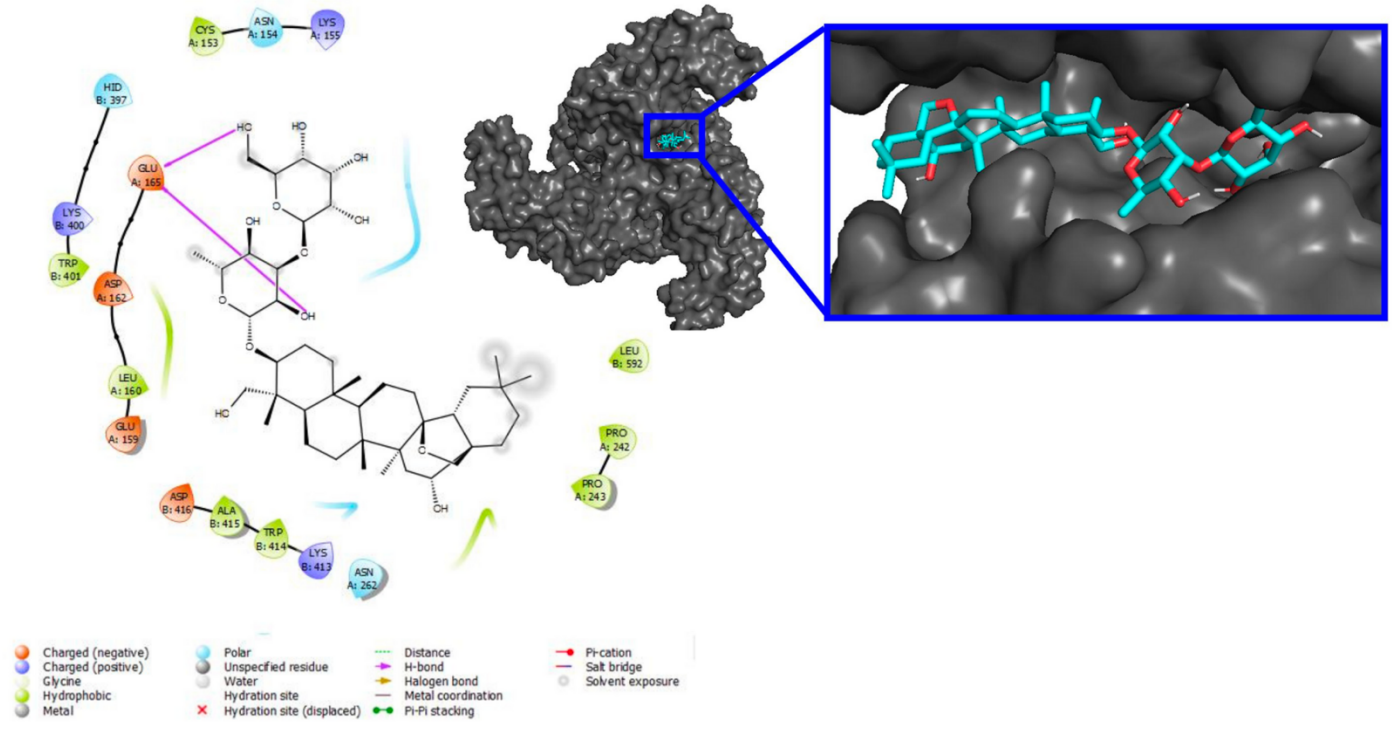


Figure 10. Interactions between Saikosaponin D and SARS-CoV-2 PLpro (6W9C)

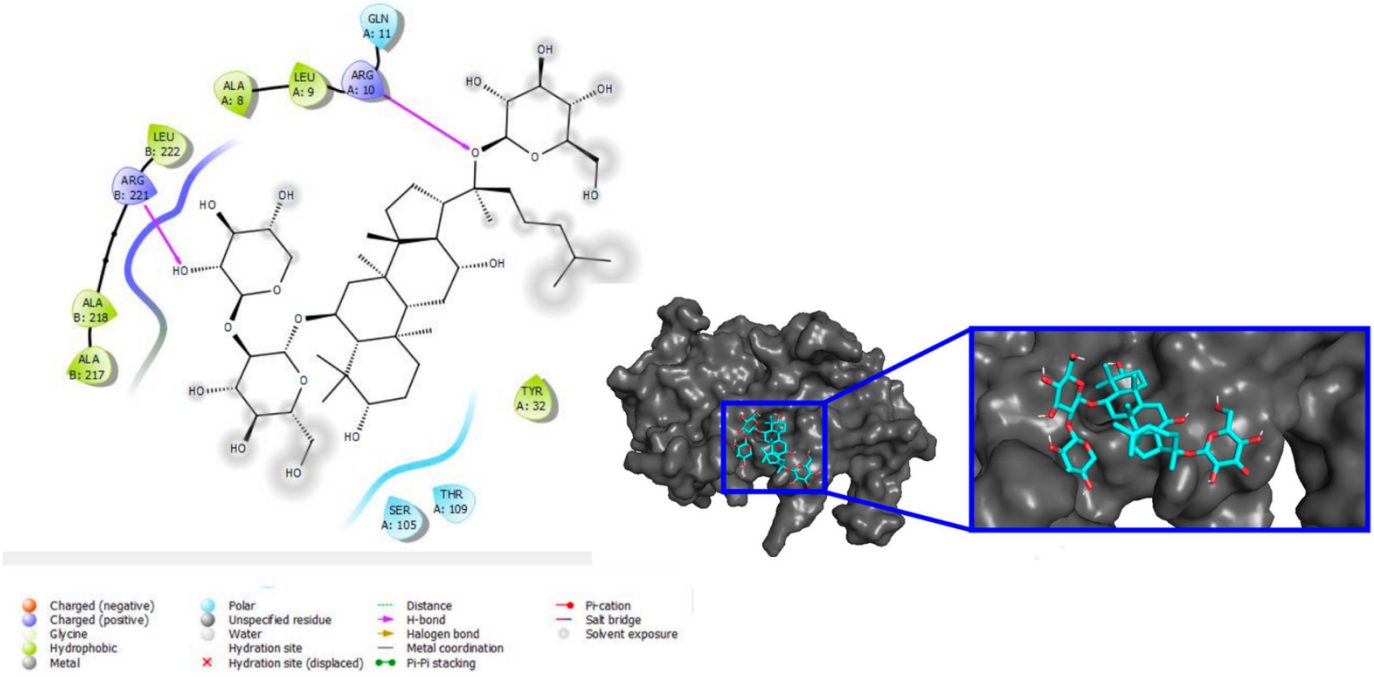


Figure 11. Interactions between Notoginsenoside R1 and SARS-CoV-2 Nsp9 replicase (6WXD)

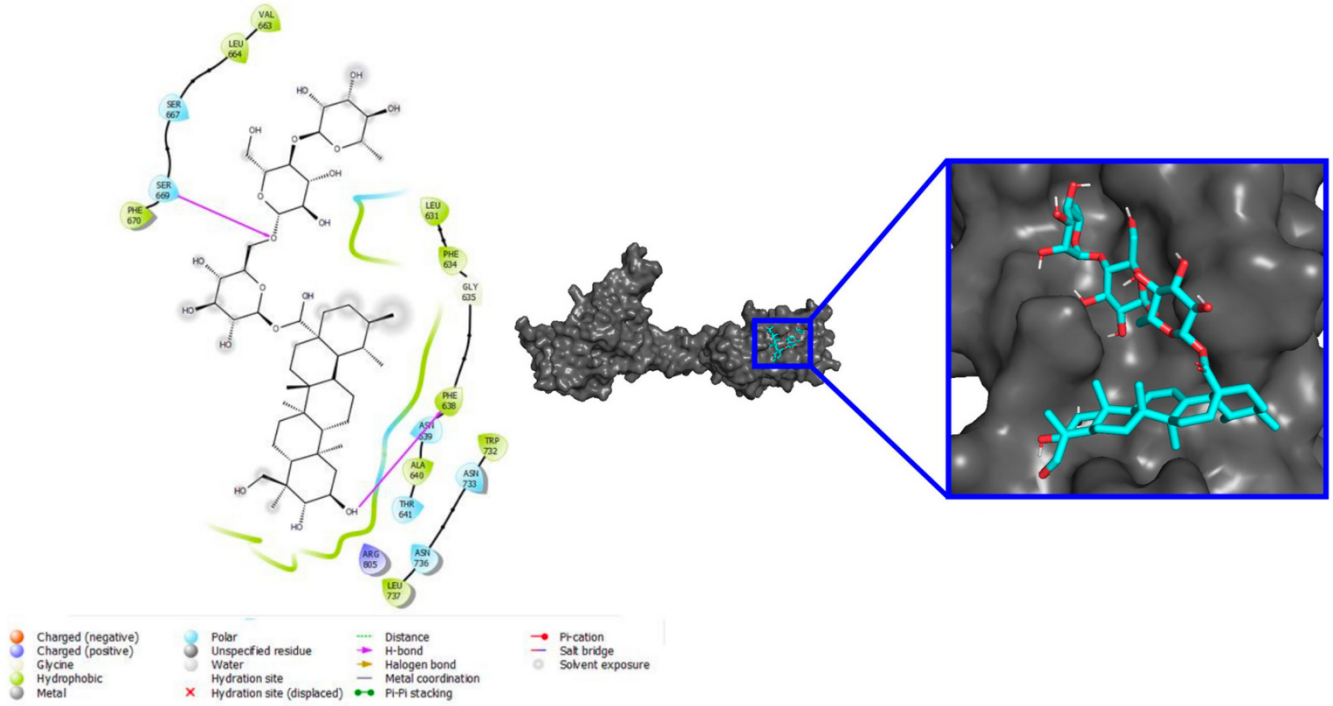


Figure 12. Interactions between Asiaticoside and SARS-CoV-2 spike glycoprotein S1 subunit (7C01)

Saikosaponin A (SSA) A, saikosaponin B2 (SSB2) and saikosaponin D (SSD) are compounds present in sickle-leaved hare's-ear (*Bupleurum falcatum*), an endemic European and Western Asian plant that has anti-ulcer properties as well as the capacity

to inhibit the growth of colon tumor cells in vitro (Sun et al., 1991; X. Zhang et al., 2022). Our molecular docking studies evince that SSA, SSB2 and SSD favorably interacted with SARS-CoV-2 ORF9b protein (6Z4U), envelope small membrane protein (7K3G) and PLpro (6W9C), respectively, exhibiting favorable binding energy scores of -8.2 kcal/mol, -8.9 kcal/mol and -9.7 kcal/mol, one by one. All H-bond interactions established between SSA and 6Z4U, SSB2 and 7K3G as well as between SSD and 6W9C can be visualized in detail on Figure 8, 9 and 10, respectively.

Notoginsenoside R1 and asiaticoside are bioactive compounds present in two traditional Chinese medicinal herbs, namely *Panax notoginseng* and Gotu Kola (*Centella asiatica*), respectively. Immune stimulation effects (Zhang et al., 2016) and alleviation of chronic hypoxic pulmonary hypertension are two *Panax notoginseng* therapeutic properties in mice (Chen et al., 2017; Wang et al., 2015). Pertaining to SARS-CoV-2, our results show that notoginsenoside R1 favorably interacted with Nsp9 replicase (6WXD), having exhibited two H-bonds with amino acid residues ARG 10 and ARG 221 (Figure 11). On the other hand, Gotu Kola is commonly used to improve scarring and to treat skin injuries, such as lupus, ulcers and psoriasis, as well as to help combat diarrhea and fever (Chen et al., 2017). In regard to the new coronavirus, asiaticoside exhibited a great binding interaction with spike glycoprotein S1 subunit (*7C01*). All H-bonds established between notoginsenoside R1 and *6WXD* as well as between asiaticoside and *7C01* can be seen in detail on Figure 11 and 12, respectively.

Only the ORF8 protein (7JTL) out of 24 SARS-CoV- 2 macromolecules did not demonstrate a favorable binding affinity with any of all chemical substances tested in our molecular docking screenings. The remarkable energy score values found in our research study involving bioactive natural compounds may be explained by means of ligand-protein stabilization through hydrogen bonds observed from our *in silico* point of view.

CONCLUSIONS

In essence, all aforementioned binding interaction profiles disclosed by our molecular docking study reveal that bioactive natural compounds derived from plants and herbs can negatively affect SARS-CoV-2 infection process. The capacity of these chemical entities to interact with essential proteins of the new coronavirus reaffirm researchers' perspective regarding the imperativeness of continuing to study phytochemicals against pathogens. Furthermore, natural compounds may be more

environmentally friendly concerning the use of solvents, for there is no need of a synthetic reaction step when it comes to extracting chemical substances. Hence, all results found in our research study indicate a valuable forthcoming application of promising bioactive natural compounds derived from plants and herbs, such as *Punica granatum*, *Centella asiatica* and *Solanum spp.*, in the realm of both in vitro and in vivo studies that will corroborate with our findings against COVID-19.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding this article.

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ANNEX I

The emerging risk of microplastics and nanoplastics on the microstructure and function of reproductive organs in mammals: A systematic review of preclinical evidence

Marcelino, R. C., Cardoso, R. M., Domingues, E. L. B. C., Gonçalves, R. V., Lima, G. D. A., & Novaes, R. D. (2022, April 15). The emerging risk of microplastics and nanoplastics on the microstructure and function of reproductive organs in mammals: A systematic review of preclinical evidence. Life Sciences. Elsevier Inc. https://doi.org/10.1016/j.lfs.2022.120404

ABSTRACT

Aims: Plastic particles (PP) pollution is a global environmental concern. Although the reproductive toxicity of PP is primarily understood for invertebrates, the evidence for mammals is still fragmented. We used a systematic review framework to investigate the reproductive impact of microplastics and nanoplastics (MNP) on mammals. *Materials and methods*: Research records were screened from Embase, Medline, Scopus and Web of Science. Twelve original papers were identified and reviewed. Immunological, oxidative and morphofunctional outcomes, and the risk of bias in all studies reviewed were analyzed. *Key findings:* These studies indicated that PP can accumulate in the gonads, triggering seminiferous degeneration, Sertoli cells death, blood-testis barrier disruption, sperm degeneration, malformation, reduced number and mobility, ovarian cysts, reduced follicular growth and granulosa cells death. Gonadal damage was associated with upregulation of prooxidant mediators (oxygen reactive species, lipid and DNA oxidation), cell death, proinflammatory molecular pathways and cytokines, as well as inhibition of enzymatic and non-enzymatic antioxidant defense mechanisms. Spermatogenesis, folliculogenesis, testosterone, progesterone and estrogen levels were also impaired in PP-treated animals, which were potentially associated with down-regulation of molecules involved in germ cells microstructural organization (occludin, N-cadherin, β-catenin and connexin 43) and steroidogenesis, such as hydroxysteroid dehydrogenases, steroidogenic acute regulatory proteins, follicle stimulating and luteinizing hormones. Selection, performance and detection bias were the main limitations identified. *Significance:* Current evidence indicates that PP can induce dose-dependent microstructural and functional gonadal damage, which is orchestrated by pro-oxidant and pro-inflammatory mechanisms that disrupt genes, molecular effectors, and hormones that control spermatogenesis and folliculogenesis.

Keywords: Animal reproduction. Experimental pathology. Plastic waste. Ovary. Testis.

INTRODUCTION

In the wake of industrial development and anthropogenic activities, we have been experiencing for decades a dramatic expansion of global production chains for plastic inputs [1,2]. Currently, 400 million tons of plastic materials are produced each year, a mass projected to double by 2050 [2]. Due to accelerated production and limited reuse, transformation or recycling initiatives, the world's ability to deal with excess of plastic products has been surpassed [3,4]. These problems are even more evident in developing countries, where plastic waste collection and treatment systems are often inefficient or unavailable [2]. As a result, the most common destination for industrial and domestic plastic waste is environmental disposal, determining extensive land and water pollution [5,6]. It is undeniable that plastic materials have a notorious relevance and applicability in the food, pharmaceutical, agrochemical, transport, construction, and electronic industries [7,8]. However, the irrational disposal of plastic products with very low shelf life (e.g., bags, packaging and bottles) often ignores the negative environmental impact of these pollutants, which can persist for decades or hundreds of years in nature causing ecological imbalances [2,9].

Once discarded in nature, plastic waste is exposed to physical (e.g., ultraviolet radiation, temperature), chemical (e.g., salinity, pH and corrosive agents) and biological (e.g., bacteria, microalgae and plankton) factors that cleave these materials into microplastic (0.1 μm–5 mm) and nanoplastic (<0.1 μm) particles [10]. In addition to being carried by water, nanoplastics are also propagated into the atmosphere from domestic and industrial dust and soot [11]. Thus, these particles already extend beyond urban centers, polluting remote environments such as inhabited alpine regions, polar ice, snow, and the deep sea [7]. This wide distribution favors the contamination of several ani- mal species with microplastics and nanoplastics, especially from integumentary exposure, inhalation and ingestion [1,11,12]. For mammals, nanomaterials inhalation and ingestion of contaminated water, sea salt and seafood represent the main routes of exposure to these plastic particles [13].

Although understood in the past as inert and safe materials, the negative biological impact of microplastics and nanoplastics contamination has recently been demonstrated [12,14]. Accordingly, smaller particles (e.g., 10 μm and 2.5 μm) penetrate organs (e.g., lungs and intestines) and cells (e.g., enterocytes and

macrophages), being recognized as foreign elements that stimulate the immune response and oxidative stress [2,15]. Due to the difficult biological clearance, the bioaccumulation of these particles can trigger chronic inflammation, favoring the development of malignancies such as cancer and death [2,16,17]. In addition, microplastics and nanoplastics also offer a high toxicological risk, since they are polymers produced from the mixture of hazardous additives such as plasticizers, flame retardants, stabilizers, colorants, antistatic agents, lubricants, slip agents, curing agents, foaming agents, and biocides [18]. These chemicals are known to be cytotoxic and act as endocrine disruptors (e.g., antiandrogens), impairing the structure of target organs, systemic metabolism, and reproductive capacity of aquatic and terrestrial animal species [19,20].

In the last decade, the evidence on the reproductive toxicity of plastic derivatives was especially concentrated on aquatic species and terrestrial invertebrates. For these organisms (e.g., Oyster, Paracyclopina nana and Eisenia fetida), exposure to microplastics and nanoplastics is often associated with activation of inflammatory and oxidative processes, genotoxicity and mutagenicity in reproductive system cells, as well as germ cell death and decreased fertility [21–24]. Despite recent advances in studies of the reproductive toxicity of microplastics and nanoplastics, knowledge about the impact of these particles on the reproductive system in mammals is recent and is still fragmented in individual initiatives around the world. Thus, it becomes difficult to map the main target organs, morphofunctional and molecular outcomes induced by this type of environmental contaminant, and assess the quality of evidence available.

Considering the growing interest in understanding the chemical- biological interactions of these plastic polymers, we used a systematic review framework to assess the impact of microplastics and nanoplastics on the structure and function of reproductive organs in mammalians. In addition to evaluating germ cells toxicity, microstructural remodeling of ovaries and testes, the mechanistic basis that associates immunological, oxidative and molecular effectors with gonadal damage and hormonal imbalance were investigated. Based on a structured screening of the methodological quality of all reviewed studies, potential risks of bias were also identified, allowing for a critical interpretation of the quality of current evidence and pointing out the main limitations that must be overcome in studies that associate reproduction and plastic by-products.

METHODS

Guiding question, databases and PRISMA workflow

Our research protocol was outlined considering the PICO/PECO strategies [25,26], which were used to outline the following guiding question: Can animals exposed to nanoplastic and microplastics develop morphological, molecular and functional reproductive damage compared to unexposed animals? To delimit the exposure factor, nanoplastic and microplastic were defined as follows: (i) nanoplastic: plastic particles smaller than 1 μm, and (ii) microplastic: plastic particles greater than 1 μm and smaller than 5 mm [27].

This study was registered in the International Prospective Systematic Review Registry (PROSPERO) under CRD registration number CRD42021277361. This systematic review was based on the updated PRISMA (Preferred Reporting Items for Systematic Reviews and Meta- analyses) guideline [28]. The PRISMA workflow was applied considering two complementary research strategies to retrieve potentially relevant studies, as follows: (i) primary search in four comprehensive electronic databases (PubMed-Medline, Embase, Scopus and Web of Sciences), and (ii) secondary search through manual screening of the reference list of all relevant studies retrieved in the primary search [29].

Search filters and search strategy

To retrieve the research records in electronic databases, a search strategy based on structured filters stratified into two complementary levels was used: (i) Biological system (reproductive system) and (ii) Exposition (nanoplastic and microplastic). Structured filters were initially developed for the PubMed-Medline search machine using standardized descriptors obtained from the MeSH (Medical Subject Headings) thesaurus and relevant indexing keywords related to the theme. Descriptors and keywords were combined by Boolean operators (AND/OR), as well as the search algorithms [MeSH Terms] and [TIAB]. Additionally, the own PubMed algorithms, “species [other animals]” and “article types [journal article]” were used in this database to get a more specific search.

The same search strategy was adapted to the Embase, Scopus and Web of Science databases using the specific syntax and search algorithms recognized by the search machine associated to each database. The filter “[Publication types - article and

article in press]" and the search limit "(Venn diagram - Sources: Embase)" were applied for Embase to restrict the retrieval of records in scientific articles and exclude duplicate studies in Medline. A similar strategy was applied in Scopus, using the algorithms "(Document type - article)" and search limit "(AND NOT INDEX (medline))". To increase the scope of our search strategy, the reference list of each relevant study identified from all databases were manually screened to identify additional potentially relevant studies. All relevant studies published up to September 2021, indexed and retrieved in full text were included in the systematic review. No chronological or language limits were applied in our search strategy. The complete search strategy and the results found are described in the supplementary files (Table S1).

Record screening and retrieval of relevant studies

Only studies investigating the reproductive effects of nanoplastics and microplastics were included in this systematic review. This selection was based on the PRISMA workflow [28,30], which was independently conducted by reviewers (R.C.M. and R.M.C.). Initially, all search records retrieved in electronic databases were loaded into the Mendeley Reference Management Program (Mendeley, London, Westminster, UK), which was used to remove duplicates by comparing indexing metadata (e.g., titles, authors, year, volume, edition, publication journal, and doi) of all databases. Then, the title and abstracts of all research records were analyzed, excluding those not related to the research topic. Then, all potentially relevant records were retrieved in full-text and evaluated for eligibility according to specific inclusion and exclusion criteria [29]. Potential disagreements at any of these steps were resolved by arbitration [31] by an expert researcher (R.D.N.). The complete PRISMA workflow obtained from our search strategy is presented in Fig. 1. The result obtained from the primary and secondary searches were compared, and the Kappa coefficient was calculated to establish the inter-rater agreement [32].

Exclusion criteria

Studies were considered irrelevant and excluded when: (i) studies that did not report the effect of nanoplastics or microplastics on the reproductive system in mammals, (ii) non-original investigations (e.g., literature reviews, editorials, letters, notes, and congress summaries), (iii) studies exclusively investigating in vitro or human systems, (iv) absence of control groups (untreated/unexposed control), (v) studies with

multiples intervention in which the effect nanoplastics or microplastics cannot be isolated, (vi) studies exclusively evaluating the bio- distribution of nanoplastics and microplastics, (vii) studies on reversible inhibition of sperm under guidance (RISUG), (viii) studies limited to nanoplastics and microplastics prenatal exposure, (ix) studies limited to styrene metabolites, and (x) grey literature (studies that have not been formally published or peer-reviewed) and (xi) studies published in languages other than English, Portuguese and Spanish. All exclusion criteria were equally applied in the primary and secondary search strategies.

Data extraction

Research data were collected from each study using extraction masks previously delimited for in vivo pre-clinical studies [33]. The following data were objectively extracted: (i) General characteristics of each study: authors, year of publication, and country in which the study was conducted; (ii) Experimental model: Animal species, lineage, sex, age, and weight; (iii) Treatment/Exposition: type and size of nanoplastics and microplastics, concentration, frequency, time and route of treatment/exposition; and (iv) Reproductive outcomes: organometric (mass of reproductive organs), microstructural/histopathological (follicles size and number, sperm count, inflammatory infiltrate, fibrosis, cell degeneration and death), inflammatory (cytokines and chemokines), oxidative (reactive species, oxidized molecules, enzymatic and non-enzymatic antioxidant effectors), biochemical (tissue and circulating levels of sexual hormones), molecular (gene expression and activation of cell signaling pathways in reproductive organs), and functional (spermatozoa motility, epididymal transit, and fertility) markers.

Risk of bias assessment

The SYRCLE's risk of bias tool was used to assess potential sources of bias in animal studies [34]. This tool is based on the Cochrane Risk of Bias (ROB) tool and was originally adjusted for specific aspects of bias that have a relevant impact on animal intervention studies. The SYRCLE's tool is structured into ten topics, which are related to potential sources of bias, such as: (i) selection, (ii) performance, (iii) detection, (iv) friction, (v) reporting and (vi) additional sources of bias not covered by other domains. The overall and individual result obtained from the SYRCLE's strategy

was graphically expressed using the Review Manager (RevMan) software, version 5.3 [35].

RESULTS

Characteristics of publications

From 600 records identified in all electronic databases, 12 relevant studies were recovered in full-text and reviewed, with 9 studies identified in the primary search and 3 from the secondary screening (Fig. 1). The list with all relevant studies identified from our search strategy is shown in Table S1. The Kappa coefficient (κ = 0.907) indicated an almost perfect agreement between the two independent reviewers who conducted the direct and indirect search for relevant studies (Table S2). Most studies identified (75%, n = 9) were developed from China. Three (25%) studies were derived from other countries such as Korea (8.33%), Pakistan (8.33%) and Iran (8.33%).

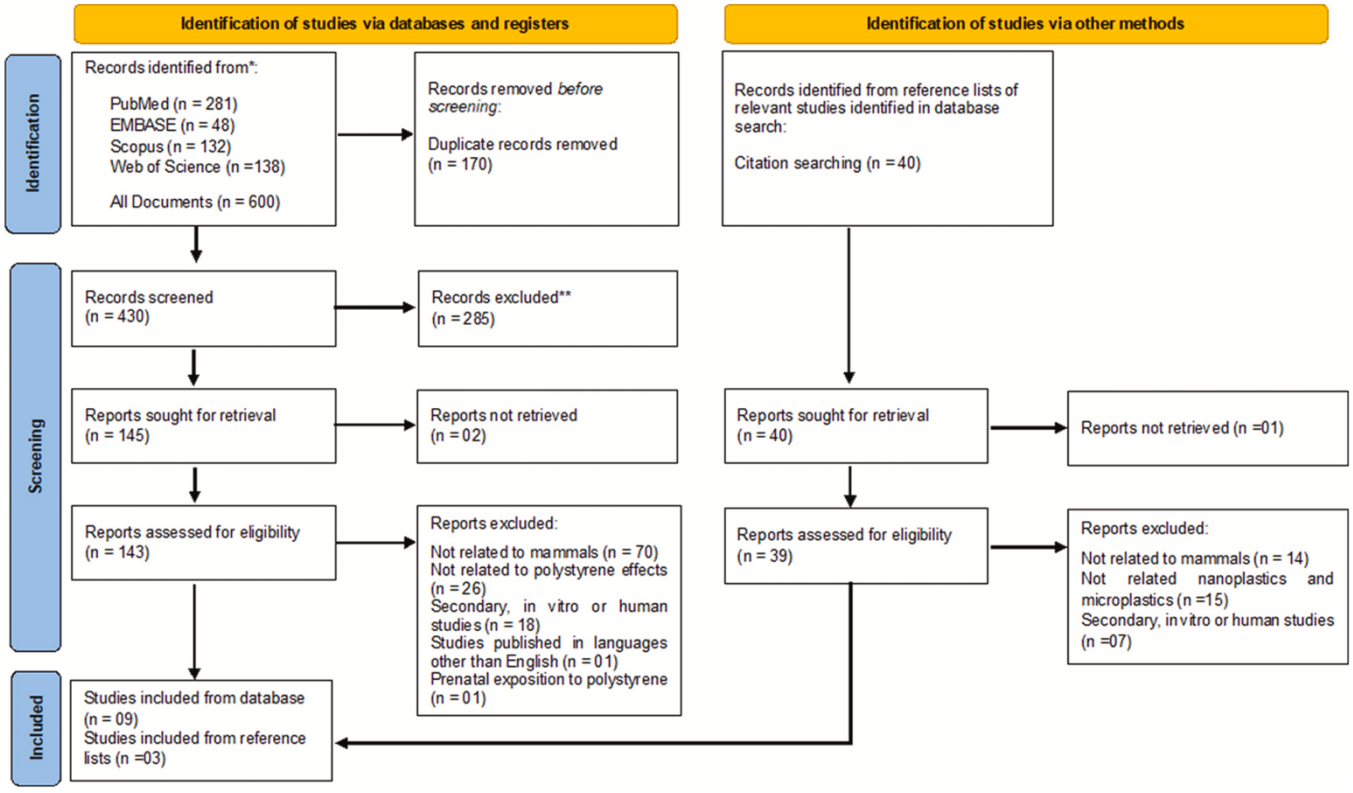


Fig. 1. Flow diagram of the systematic review literature search results. Based on PRISMA statement "Preferred Reporting Items for Systematic Reviews and Meta-Analyses". www.prisma-statement.org.

Characteristics of experimental animals

As shown in Table S3, 7 studies (58.33%) used mice and 5 (41.66%) used rats as animal model. Wistar rats and BALB/c mice were the main lineages used in these

studies and represent 33.33% of each lineage adopted. Two studies (16.66%) reported ICR mice, while CD-1 mice and Sprague Dawley rats were reported in 1 study each (8.33%). Most studies (75%, n = 9) used male animals, and females animals were adopted in 2 papers (16.66%). Male and female animals were used in only 1 study (8.33%). Mice weight was reported in 1 study (8.33%), with ranging from 20 g–25 g. Rats weight of was reported in 4 studies (33.33%), ranging from 170 g–190 g. Animals age of animals range from 28 to 60 days (Table S3).

Characteristics of microplastic and nanoplastic treatments

As indicated in Table 1, a similar proportion of studies investigating nanoplastics and microplastics was identified (n = 6, 50.00% each). At the nanometer scale, the size of plastic particles ranged from 0.5 nm to 100 nm, while micrometer particles range from 0.4 μm to 48 μm. Plastic particles were administered by gavage in 9 studies (75.00%), and 3 studies (25.00%) administered these particles ad libitum in drinking water. The time of treatment with plastic particles ranged from 28 days to 90 days. The gonadal accumulation of plastic particles was investigated and proved by biofluorescence or transmission electron microscopy in 6 studies (50.00% each).

Effect of microplastic and nanoplastic on morphological outcomes in reproductive structures

As detailed in Table S4, a broad spectrum of microstructural changes was identified in male reproductive system. In general, microplastic and nanoplastic was associated to testicular atrophy [14,36], vacuolization of seminiferous epithelium [36–38], degeneration of the seminiferous tubules/epithelium [14,37–42], disorders/incomplete spermatogenesis, Sertoli cells microstructural damage [14,43], and disorganization/ disruption or increased permeability of the Sertoli cells barrier (blood- testis barrier) [14,37,38,41], reduced number of germ cells [37,39,40,44], accumulation of immature germ cells [36], germ cells with pyknotic nuclei and kariorhexis [39], germ cells abscission [41], and inflammatory infiltrate in seminal vesicle [42]. Considering the male gametes, the main microstructural changes detected were: Sperm acephalia, tailless, small head, acrosome loss [41], curled or folded tails, no tail shape, spliced sperm with double head and double tails, curled or folded heads, head without hook, and sperm swollen [14,37,39,44], reduced sperm

mobility or immobility [40,41] and apoptosis [40], reduced sperm count [38,39,44] (Table S4).

In female reproductive system, the main microstructural abnormalities identified were: Dilatation in fallopian tubes, presence of ovarian cysts and increased number of corpus luteum. [42], reduced thickness of the granulosa layer in secondary follicles [45], reduced number of growing follicles, increased ovarian collagen and fibronectin accumulation [46], apoptosis of granulosa cells [45,46] (Table S4).

Table 1
General characteristics of the plastic particles (PP) and treatments administered in all studies included in the systematic review.

Study	Species	Plastic particles size	Sex	Treatment protocol	PP gonadal accumulation
Amereh et al. [14]	Rats	0.5 nm	Male	Control group: distilled water Treated groups: 1, 3, 6, 10 mg kg-bw/day Administration: gavage Duration: 35 days	Yes (Biofluorescence)
Deng et al. [43]	Mice	0.4–0.5 μm	Male	Control group: Treated groups: 1, 3, 6, 10 mg/kg-bw/day Administration: gavage Duration: 35 days	Yes (Transmission Electron Microscopy)
Park et al. [42]	Mice	40–48 μm	Male/female	Control group: (–) Treated groups: 3.75,15, 60 mg/kg-bw/day Administration: gavage Duration: 90 days	NI*
Xie et al. [44]	Mice	5.0–5.9 μm	Male	Control group: saline Treated groups: 0.01, 0.1, 1 mg/day Administration: gavage Duration: 42 days	NI*
Hou et al. [39]	Mice	5 μm	Male	Control group: saline Treated groups: 0.6–0.7, 6–7, 60–70 μg/day Administration: ad libitum Duration: 35 days	NI*
Hou et al. [45]	Mice	0.5 μm	Female	Control group: deionized water Treated groups: 0.015, 0.15, 1.5 mg/kg/day Administration: ad libitum Duration: 90 days	Yes (Transmission Electron Microscopy)
An et al. [46]	Rats	0.5 μm	Female	Control group: deionized water Treated groups: 0.015, 0.15, 1.5 mg/day Administration: ad libitum Duration: 90 days	Yes (Transmission Electron Microscopy)
Ijaz et al. [40]	Rats	10 μm	Male	Control group: saline Treated groups: 2, 20, 200, 2000 μgL^{-1}/day Administration: gavage Duration: 60 days	NI*
Jin et al. [41]	Mice	0.5, 4 and 10 μm	Male	Control group: deionized water Treated groups: 10 mg/mL Administration: gavage Duration: 28 days	Yes (Biofluorescence)
Wei et al. [38]	Mice	4 μm, 10 μm	Male	Control group: (–) Treated groups: 20, 40 mg/kg Administration: gavage Duration: 28 days	NI*
Xu et al. [36]	Mice	100 nm	Male	Control group: deionized water Treated groups: 1 mg/mL Administration: gavage Duration: 28 days	Yes (Biofluorescence)
Li et al. [37]	Rats	0.5 μm	Male	Control group: deionized water Treated groups: 0.015, 0.15, 1.5 mg/kg Administration: gavage Duration: 90 days	NI*

(-) Data not reported or incomplete information. NI* *Data not investigated. (nm) Nanometer. (μm) Micrometer. (mg) Milligram. (kg) Kilogram. (bw) Bodyweight. (μg) Microgram.*

Effect of microplastic and nanoplastic on inflammatory, oxidative and hormonal effectors

As detailed in Table S5, microplastic and nanoplastic trigger pro- inflammatory and pro-oxidant events, as well as imbalance in sexual hormones levels in male and female animals. Considering inflammatory effectors, the treatment with these plastic particles upregulated TNF-α, IL-1β and IL-6 [36,39,41,44], IL-1β and IL-18 [45], MCP-1 and CXCL10 [41] and p-NF-κBp65 levels [39,45], and reduced IκBα levels [39,45].

The analysis of redox markers indicated that plastic particles upregulated sperm oxidative DNA damage [14], MDA and ROS [37,43–46], ACP, LDH and SOD [43], and down-regulated Nrf2 and HO-1 levels [38], SDH [43,44], LDH, GSH-PX [44], CAT, GSH-PX and SOD enzyme levels and/or activity [37,40,45,46] (Table S5).

The hormonal panel indicated consistent down-regulation in testosterone [14,40,41], LH [14,40], FSH [40], and AMH levels [45,46] (Table S5).

Effect of microplastic and nanoplastic on gene expression and gonadal regulatory proteins

Molecular screening based on real-time polymerase chain reaction (PCR), western blotting and immunohistochemistry indicated extensive gonadal molecular dynamics induced by treatment with microplastics and nanoplastics (Table S6). Accordingly, microplastics and nano- plastics gonadal exposure upregulated Bik [14], Bax [40] gene expression. In addition, increased p-JNK, p-p38 MAPK [37,44], caspase 3 [36,40,44,45], Bax [37,39,46], cleaved caspase 1, NLRP3 [45], Wnt, β-catenin, and p-β-catenin [46] protein levels.

Conversely, these plastic particles down-regulated ABP, PLZF, DAZL [14], INSL3 [43], 3β-HSD, 17β-HSD, StAR and Bcl2 [40] gene expression. In addition, attenuated Bcl2 [37,39,46], occludin, N-cadherin [37,38,41], zonula occludens 1, β-catenin [38,41], FAK [41], connexin 43 [37,38], Arp3, Eps8 [38], and claudin-11 [37] protein levels (Table S6).

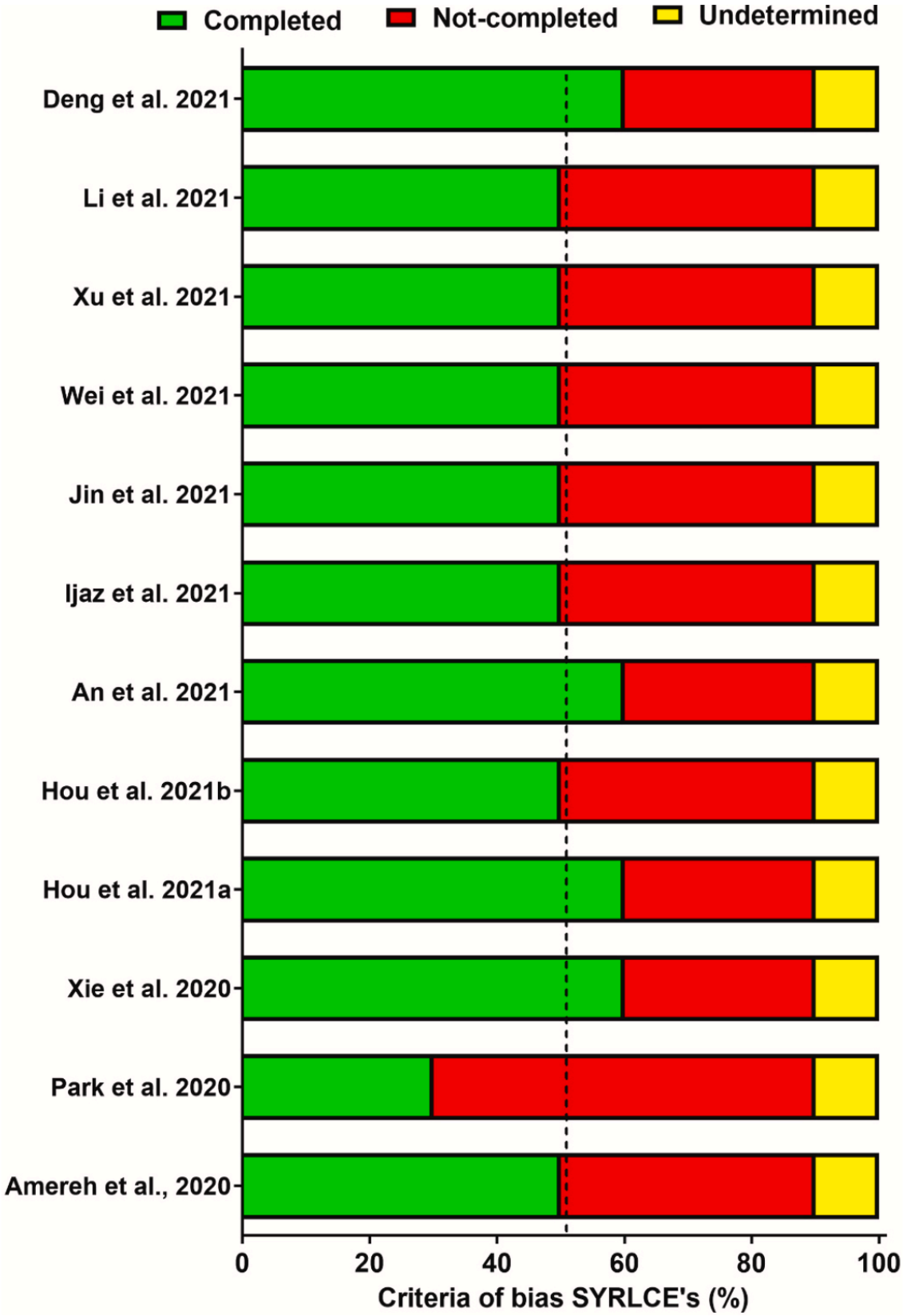


Fig. 2. Analysis of the risk of bias in each study included in the systematic review. Based on the SYRCLE's risk of bias tool for animal studies. The dotted line indicates the average score obtained for all studies reviewed.

Risk of bias

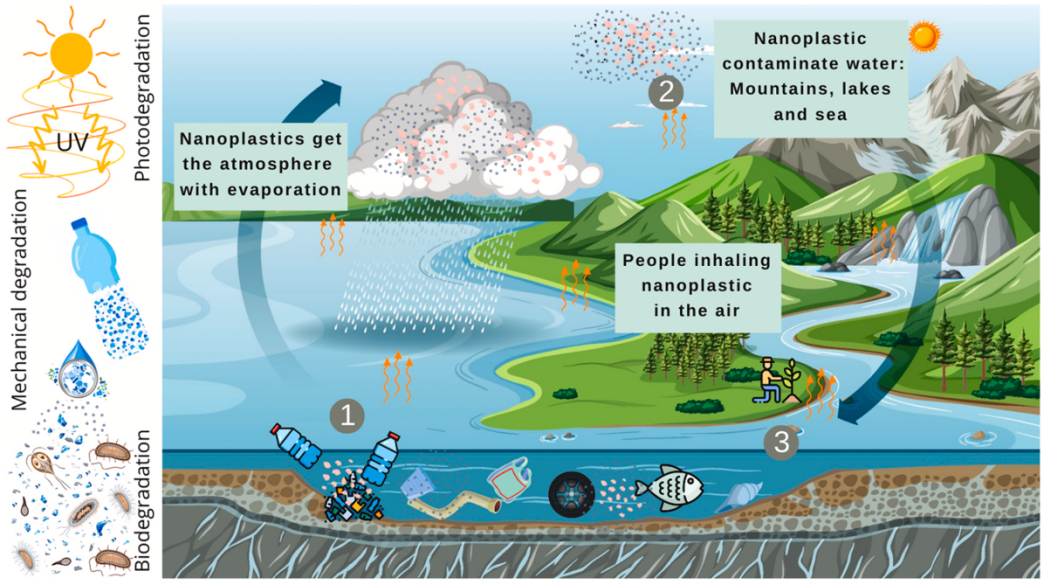
The detailed results of the bias analysis based on the SYRCLE's tool are detailed in Table S7 and synthesized in Fig. 2. No study reviewed met all criteria for methodological quality, indicating potential risks of bias in different domains evaluated. Methodological quality aspects such as animal allocation sequencing, investigator blinding of treatment groups, and rater blinding during data collection were underreported in all studies. Selection of animals for random data collection was performed in only 4 studies (33.33%). Baseline characteristics and random housing of animals were met by most studies (91.6%, n = 11). Incomplete outcome data, selective outcomes, and other potential bias sources (e.g., types of plastics, particle sizes and administration routes) were adequately addressed in 12 studies (100%). Taken together, the reviewed studies achieved a 51.65% mean score for the risk of bias. Only 4 studies (33.33%) exceeded the mean score.

DISCUSSION

Motivated by growing concern about the biological impact of plastic waste pollution, we have identified recent evidence that plastic particles can induce marked dose-dependent reproductive toxicity in mammals. Despite the wide geographic distribution of plastic waste, available evidence on the reproductive toxicity of microplastic and nanoplastic is concentrated in research initiatives from Asian countries. Accordingly, China leads these investigations, a feature consistent with the highest production and disposal indicators for plastic waste worldwide ([1,2,5] [39,45]).

From the environmental accumulation of plastic waste, biotic and abiotic elements such as solar radiation, heat, pH, salinity, and biofilm formation promote the breakdown of plastic materials into micrometric and nanometric particles [47,48], which present a wide flow across reservoir ecosystems (Fig. 3). Accordingly, plastic nanoparticles can disperse in the atmosphere from water evaporation, representing a route of inhalation contamination [11]. Furthermore, water precipitation spreads plastic contamination to mountains, lakes and oceans [16,47,48]. When using this water, animals and humans are contaminated, and processes of trophic transfer and biomagnification can also occur along the food chain from the ingestion of products contaminated with these plastic particles (e.g., seafood) [16,47,48] (Fig. 3 and Fig. 4). Notably, the toxicological concern associated with contamination by plastic particles

goes beyond its direct effect in obstructing the digestive tract and impairing nutrient absorption [36,49,50]. Although this obstruction is more frequent in aquatic species, the release of chemicals associated to plastic polymers is a central mechanism of toxicity in mammals [51,52] (Fig. 4). Thus, testicular dysgenesis, cancers, follicle loss and decreased fertility in male and female mammalians has been associated with styrene, bisphenol A, phthalate esters, organophosphates, hydroxytoluene and aromatic hydrocarbons [38,43,52], which are common toxicants present in plastic composition [19,20].



1. Plastic materials releasing fragments and chemical compounds on the environment. **2.** Nanoplastic in the atmosphere. **3.** Plastic-derived chemical compounds contaminating animals and humans.

Fig. 3. Biogeochemical cycle of plastic particles. This model considers a continuous and complex dynamics of plastic materials in different environmental matrices, affecting several animal species, including humans. Photodegradation is mainly represented by ultraviolet (UV) radiation from sunlight. Mechanical degradation is especially mediated by shear, tension and compression forces. Biodegradation included the fragmentation catalyzed by biomolecules released by bacterial biofilms, aquatic and terrestrial organisms.

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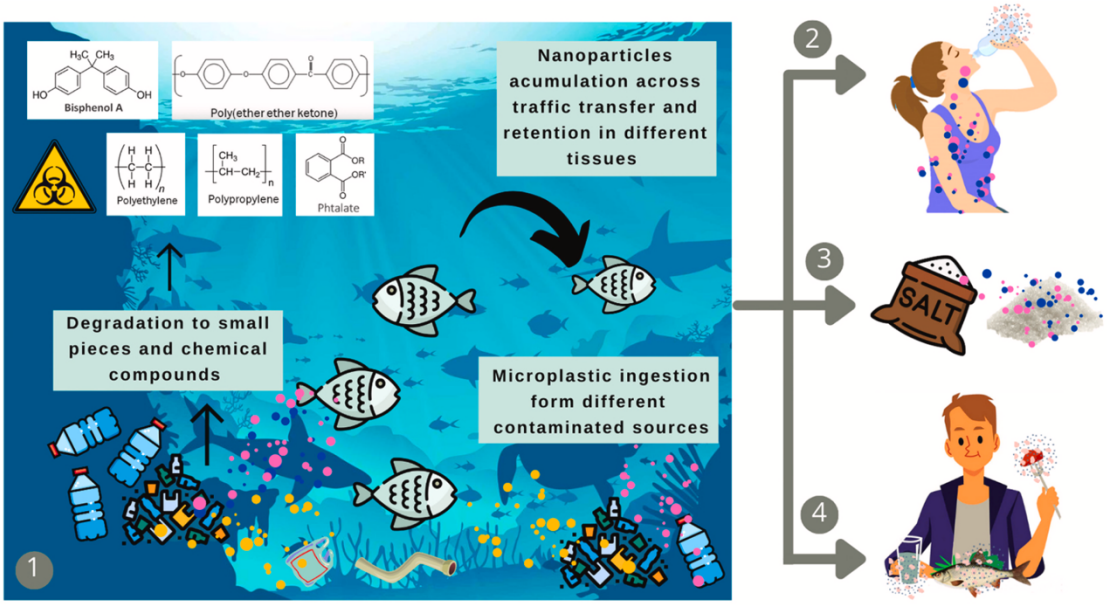
Despite plastic pollution being a global phenomenon with negative impact on several ecosystems, we identified that evidence on reproductive toxicity in mammals was limited to murine models. This finding is consistent with the lower cost and greater ease of acquisition, easier handling and better control of experimental conditions [36,40,46]. Although plastic residues can affect several species of mammals, studies

with wild species are subject to greater regulatory scrutiny linked to animal welfare (e.g., capture and management practices), limiting the use of animal models different from those adopted in the laboratory routine [53,54]. As the regulatory mechanisms of reproduction are highly conserved in mammals [55,56], it is not possible to rule out that the alterations identified in rodents can also manifest in other species. Thus, further studies are essential to map the resistance and/or susceptibility of different mammalian species to plastic residues.



1. Plastic particles and their toxic chemicals bioaccumulate in aquatic organisms. **2.** People drinking water contaminated with micro- and nanoplastics. **3.** Salt contaminated with micro- and nanoplastics. **4.** Humans eating seafood with micro- and nanoplastics.

Fig. 4. Representative model microparticles and nanoparticles formation, release of toxic compounds from plastic waste, traffic transfer across alimentary chain and the main sources of oral contamination from water, salt and seafood intake. In this model, humans were used to represent the contamination of mammals with plastic particles. Only a few toxicants present in the composition of several plastic polymers were represented in this model.

Fortunately, all the studies reviewed evaluated oral exposure to plastic particles, which is the usual exposure route in mammals and the most frequent form of environmental contamination with these residues [14,42,44]. Furthermore, most studies indicated that plastic particles exerted a dose-dependent negative effect on reproductive organs [14,37–41,45,46]. However, administration by gavage or free access to drinking water indicates variable experimental control of plastic particles exposure, which may limit the characterization of dose-dependent responses due to the difficulty in ensuring the amount of particles ingested in ad libitum exposure models [39,45,46]. Unfortunately, ingestion of these plastic particles was evaluated for short periods (28–90 days), restricting the available evidence to the acute reproductive toxicity.

In general, plastic particles were detected in male [14,36,41,43] and female [45,46] gonads. This finding was coupled with extensive microstructural damage, especially degeneration of the seminiferous epithelium [14,36–42], Sertoli cells death and disruption of the blood-testis barrier integrity [14,37,38,41,43], sperm malformation or degeneration [14,37,39,41,44], reduced sperm count [38,39,44] and reduced sperm motility [40,41]. In addition, ovarian cysts [42], reduced number of growing follicles [46] and apoptosis of granulosa cells [45,46] were identified in female gonads. Current evidence indicates that plastic particles less than 5 to 10 μm can cross the stomach and intestinal epithelium via paracellular and transcellular pathways, allowing its systemic distribution [49,50]. Thus, the detection of plastic particles in testis [14,36,41,43] and ovary [45,46] was consistent with this gastrointestinal translocation, whose size-dependent systemic distribution was also identified in mice orally treated with 0.01, 0.1 and 0.5 mg plastic particles (5 μm and 20 μm diameter) for up to 28 days [57]. Apparently, the intestinal permeability and particles translocation can be potentiated by damage to the intestinal epithelium (Fig. 5), including the death of enterocytes, which can be mediated by inflammatory and oxidative processes triggered by plastic particles [44,46,49].

Experimental findings also suggested that plastic particles can translocate across living cells to the lymphatic system [13,58], diffuse through blood capillaries [14], and be captured by macrophages [13,49]. Thus, these particles have the potential to reach the gonads (Fig. 5), disrupting the blood-testicular barrier [14,37,38,41] and the follicular development [46]. Most particles larger than 10 μm are trap- ped and then eliminated by the digestive tract [50]. However, they cannot be considered inert, since can release oligomers or toxic molecules from chemical degradation [13,50], which can also be catalyzed by oxygenase enzymes produced by some intestinal bacteria [50]. Many of these compounds, especially bisphenol A, phthalates, heavy metals and brominated flame retardants exhibit particular toxicological interest as they act as potent endocrine disruptors, proinflammatory and prooxidant agents [19,20,51].

From a mechanistic approach, the reviewed studies reinforced the evidence linking dose-dependent gonadal damage to the inflammatory and redox imbalance attributed to chemicals present in plastic composition. Accordingly, plastic particles upregulated prooxidant pathways (e.g., ROS production, lipid and DNA oxidation) [14,37,43–46] and inhibited antioxidant defenses (e.g., Nrf2, HO-1, SDH, LDH, GSH-

PX, CAT and SOD) [37,40,43–46]. This response is potentially associated with intracellular accumulation of plastic particles and lysosomal instability, directly releasing ROS or ROS-catalyzing enzymes in the interstitial compartment [59]. In addition, the gonadal oxidative stress induced by this plastic products was closed correlated to NF-κB [39,45], JNK, p38 MAPK [37,44], Wnt and β-catenin activation [46]. As these pathways modulate cytokines and chemokines production [60–62], the upregulation of proinflammatory effectors (e.g., TNF-α, IL-1β, IL-6, IL- 1β, IL-18 and MCP-1 and CXCL10) was consistently implicated in plastic particles-induced gonadal damage [36,39,41,44]. Interestingly, inflammatory and oxidative damage were potentiated in animals treated with higher doses of plastic particles [37,39,40,45,46]. However, these dose-dependent effects cannot be attributed to the gonadal accumulation of these particles. As the current evidence does not clarify the relationship between the administered dose and the tissue load of plastic particles, regional and systemic effects cannot be ruled out to explain this dose-dependent behavior, an issue whose understanding requires further mechanistic investigations.

As expected, the prooxidant and inflammatory responses were associated with a marked dose-dependent upregulation in cell death effectors (e.g., Bik, Bax, caspase 1 and 3) and down-regulation in genes and proteins involved in cell survival (e.g., Bcl2), spermatogenesis and postnatal folliculogenesis (e.g., PLZF, DAZL, INSL3, occludin, N-cadherin, zonula occludens 1, β-catenin, FAK, and connexin 43) [14,37,39,40,43,46]. Most of these molecules are not essential for mammalian fertility; however, regulates the reproductive function by controlling blood-testis-barrier integrity, germ cells structure, communication, proliferation and differentiation [55,56]. Thus, the depletion of connecting (e.g., FAK, claudins, occludins and cadherins) and communication (e.g., connexin) molecules breaks Sertoli cells barrier and the adluminal microenvironment required for a proper spermatogenesis [63,64]. This down-regulation also interrupts the physical- functional interactions between ovarian stroma and parenchyma, compromising oocytes-granulosa cells synchronism and postnatal folliculogenesis [65,66]. Conversely, depletion DAZL protein has been directly associated with infertility in male and female rodents and humans [67,68].

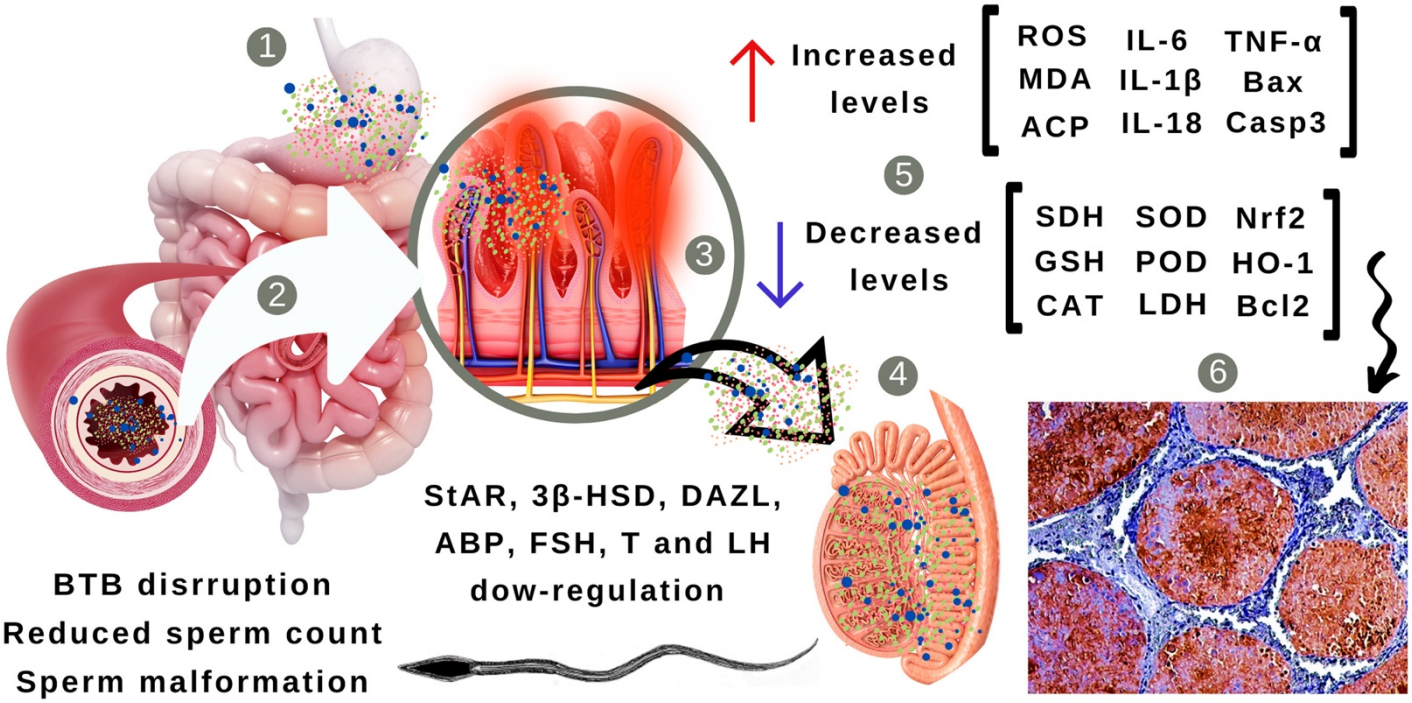


Fig. 5. Representative model of plastic particles gastrointestinal absorption, systemic distribution, testis accumulation, biochemical and microstructural damage. (1) Ingestion and plastic particles in the stomach (1) and intestine (2) of mammals. (3) Intestine inflammation and plastic particles through the lining epithelium. (4) Systemic distribution and testis accumulation of plastic particles. (5) Upregulation of prooxidant and proinflammatory molecules and down-regulation of antioxidant effectors in testis. (6) Degeneration of the seminiferous epithelium with germ cells death (apoptotic cells marked in brown color). ROS = reactive oxygen species, MDA = malondialdehyde, ACP = acid phosphatase, IL = interleukin, TNF-α = tumor necrosis factor alpha, Bax = BCL2 associated X, apoptosis regulator, Casp3 = caspase 3, SDH = succinate dehydrogenase, GSH = *glutathione*, CAT = catalase, SOD = superoxide dismutase, POD = peroxidase, LDH = lactate dehydrogenase, Nrf2=, HO-1 = heme oxygenase-1, Bcl2 = B-cell lymphoma-2 apoptosis regulator, FSH = follicle stimulating hormone, LH = luteinizing hormone, T = testosterone. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Strikingly, plastic particles toxicity has extended to endocrine disruption. Thus, animals receiving these particles manifested reduced testosterone [14,40,41,44], progesterone and estrogen levels [40], which were related to dose-dependent down-regulation of genes and/or enzymes involved in steroidogenesis, such as FSH, LH, ABP, 3β-HSD, 17β-HSD and StAR [14,40,45,46]. These changes indicate regional and systemic plastic-induced toxicity on hormonal regulation of reproductive function. As gonadal structure and function are dependent on adequate steroidogenesis [14,40,56], its inhibition may be associated with down-regulation in the expression of gonadal

regulatory molecules and microstructural damage identified in the reviewed studies. Thus, reproductive toxicity of plastic particles involves multiple gonadal tar- gets associated to dose-dependent prooxidant, proinflammatory, and hormonal events that orchestrate seminiferous epithelium and blood- testis barrier disruption, sperm morphofunctional degeneration, germ cells death and follicular degeneration.

Considering a critical interpretation of the evidence, the assessment of methodological quality indicated important elements of bias in the reviewed studies. Even considering the specificities of each research design, no study fulfilled all methodological criteria. The studies presented variable methodological score without a temporal influence (year of publication), indicating that elements of bias are continuously replicated despite methodological advances and availability of more sensitive and specific analytical tools. Surprisingly, 40% of the essential criteria to be reported in in vivo animal studies were neglected. Underreported aspects, such as animals' allocation sequence and concealment (allocation, treatment groups and data collection) under- mine the reproducibility, internal and external validity of the reviewed studies, limiting evidence reliability [34]. Conversely, the description of animals' randomization, baseline characteristics (characterization of models and experimental conditions), outcome data adequately addressed, absence of selective outcomes, and complete characterization of plastic particles treatments represented the criteria broadly met by the reviewed studies. It is important to emphasize that these bias elements do not indicate flaws in the experimental protocols, they only point out limitations in the research report. Thus, by mapping the risk of bias in all investigated studies, this review provides objective support to delimit further studies with greater methodological rigor, providing unequivocal evidence on the reproductive impact of plastic particles in mammalians.

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DECLARATION OF COMPETING INTEREST

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

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