In silico targeting cysteine protease 2 of *Giardia lamblia* by *Origanum vulgare* L. flavonoids as potential inhibitors

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Abstract

Giardiasis, caused by *Giardia lamblia*, is a prevalent and problematic infection. Current treatments, such as metronidazole have some limitations. Flavonoids may possess anti-giardia properties. As CP2 (giardipain-1) plays a critical role in the parasite's pathogenicity, the present study aimed to discover if the flavonoids of marjoram can target CP2 and inhibit it. After modeling the CP2 spatial structure and obtaining the chemical structure of flavonoids, molecular docking was performed using PyRx. Subsequently, pharmacokinetics and the toxicity of flavonoids with the highest binding affinity for CP2 were investigated. Finally, molecular dynamics simulation was conducted on CP2 and the final candidate. Among marjoram flavonoids, isovitexin, cosmosiin, and apigenin 7-*O*-methylglucuronide exhibited the highest binding affinity for CP2. However, toxicity studies revealed that isovitexin and cosmosiin are mutagens. Therefore, only apigenin 7-*O*-methylglucuronide can be considered a potential drug for treating giardiasis. Nevertheless, further studies are needed to confirm this hypothesis.

Keywords:

Flavonoids
Molecular dockings
Cysteine proteases
Giardia lamblia
Marjoram

1. Introduction

Giardiasis is the most common intestinal protozoan infection caused by *Giardia lamblia*. While in developed countries, approximately 2.0% of adults and 8.0% of children are infected with the parasite, in developing countries, approximately 33% of the population have giardiasis (Zajaczkowski et al., 2018). Watery diarrhea and bloating are common intestinal symptoms of this infection (Vivancos et al., 2018). In addition, chronic infection may cause weight loss and vitamin deficiency (Cordingley and Crawford, 1986; Girard et al., 2006).
CP2 (giardipain-1) is a pivotal cysteine protease in *Giardia lamblia*, playing a crucial role in the parasite's pathogenicity (Quezada-Lázaro et al., 2022). It facilitates host cell invasion by breaking down extracellular matrix proteins, enables nutrient uptake by degrading host proteins, and aids in immune evasion by disrupting immune molecules and signaling pathways (Allain et al., 2019; Ortega-Pierres and Argüello-García, 2019; Argüello-García et al., 2023). These essential functions make CP2 a promising target for therapeutic intervention against giardiasis.
Metronidazole is the first-line treatment for giardiasis. However, taking this medicine can have various side effects. Some of the side effects of metronidazole are nausea, abdominal pain, diarrhea, neurotoxicity, optic neuropathy, peripheral neuropathy, and encephalopathy. Meanwhile, researchers have suggested that metronidazole and its metabolites can bind to RNA and inhibit protein synthesis, leading to axonal degeneration in nerve fibers. In addition, the use of this drug in pregnant women, especially in the first trimester of pregnancy, may cause cleft lip (Alston and Abeles, 1987; Rao and Mason, 1987; Freeman et al., 1997; Dingsdag and Hunter, 2017; Goolsby et al., 2018).
Marjoram (*Origanum vulgare*) is a medicinal plant in *Lamiaceae* family. Members of this family, such as the genera *Perovskia* and *Marrubium*, have been extensively studied for their rich phytochemical composition and bioactive properties. Studies on the genus *Perovskia* have identified over 40 novel natural compounds with diverse biological activities, further emphasizing the medicinal potential of *Lamiaceae* plants (Mohammadhosseini et al., 2021). Furthermore, the genus *Marrubium* has been explored for its essential oils and volatiles, revealing significant chemical diversity and pharmaceutical potential (Mohammadhosseini,

2016). These findings underscore the importance of continued exploration of the *Lamiaceae* family for novel compounds and therapeutic applications (Camilo et al., 2017). In traditional medicine, marjoram has been employed as an anti-asthma, anti-spasm, and anti-anxiety agent. Additionally, it is used to address various digestive disorders such as stomach and intestinal issues, constipation, and flatulence (Kordali et al., 2008). Laboratory investigations have further highlighted the plant's beneficial properties, including antioxidant, anticancer, antimicrobial, and anti-inflammatory effects. Moreover, marjoram has shown potential in reducing cholesterol and blood sugar levels (Chaudhry et al., 2007; Saeed and Tariq, 2009; Kaurinovic et al., 2011). The phytochemical profile of marjoram includes flavonoids, tannins, glycosides, sterols, vitamins, and terpenoid compounds. Other species of the *Lamiaceae* family also have flavonoid (Mohammadhosseini, 2016). Flavonoids, in particular, are significant phenolic compounds in the human diet, known for their antioxidant, anti-inflammatory, and antiviral activities (Wang et al., 2018; Al-Khayri et al., 2022).

Phytochemicals of medicinal plants are an excellent source for drug design and discovery (Parvizpour et al., 2021). With the increasing prevalence of giardiasis and the limitations of current treatments, the need for novel, effective, and cost-efficient plant-derived anti-giardiasis drugs is more urgent than ever. Multiple plant compounds have been developed into anti-giardiasis medications in the past decade, and about a quarter of plant-derived agents have been approved by the FDA and EMA, highlighting the value of phytochemicals in the pharmaceutical industry. These advancements underscore the importance of your work in developing new treatments for giardiasis (Patridge et al., 2016).

Identifying anti-giardiasis phytochemicals faces some obstacles, but we have powerful tools at our disposal. Computational tools in pharmacoinformatics, such as molecular docking, virtual screening, and molecular dynamics simulation, have streamlined drug design from compound characterization and target identification to screening and repurposing. These methods have proven to be effective in discovering, designing, and analyzing new drug candidates, and we are confident in their potential to advance our understanding of giardiasis treatment (Kontoyianni, 2017; Wu et al., 2020; Salo-Ahen et al., 2021).

Previous studies have shown that flavonoids of different plants may exhibit antiprotozoal activity (Calzada et al., 1999; Hernández-Bolio et al., 2015; Ticona et al., 2022). On the other hand, an *in vitro* study by Dawoodi et al. showed that *O. vulgare* extract has anti-giardiasis activity (Davoodi and Abbasi-Maleki, 2018).

Considering the importance of CP2 in the pathogenesis of G. *lamblia*, the observed anti-giardia effects of the marjoram plant may be partially via the inhibitory effects of its flavonoids on this enzyme. Therefore, the present *in silico* study aimed to investigate the possible inhibitory effects of marjoram flavonoids on *G. lamblia* CP2 using the molecular docking method.

2. Experimental

2.1. Protein preparation and validation

In this study, CP2 (cysteine protease 2, Giardipain-1) of *G. lamblia* was selected due to its critical role in the pathogenic effects of the parasite. The amino acid sequence of CP2 was taken from the UniProt database. In the next step, the three-dimensional structure of the proteases was searched in the PDB database. The three-dimensional structure of CP2 was modeled using the SWISS-MODEL online web server. Different analysis programs, namely VERIFY 3D, PROCHECK, ERRAT, and PROSA, were employed to evaluate the quality of the predicted structural model of CP2. The ERRAT program validates the predicted model by statistically analyzing the non-bonding interactions between different types of atoms (Colovos and Yeates, 1993). PROCHECK checks the structure of proteins by analyzing the side chains' geometry and the model's overall geometry using the distribution of Phi/Psi angles in the Ramachandran plot (Laskowski et al., 1993). VERIFY 3D determines the position and compatibility of the atomic model with the corresponding amino acid sequence and compares it to the appropriate structure (Eisenberg et al., 1997). Finally, the PROSA program evaluates the overall quality and identifies the potential errors or irregularities in the protein structures. In this respect, it provides a comprehensive analysis of protein structures (Wiederstein and Sippl, 2007).

2.2. Ligand obtaining and preparation

Marjoram flavonoids were obtained from three different databases. The first was IMPPAT, which contains a large amount of data on medicinal plants and their phytochemicals, with over 1700 Indian medicinal plants and 9500 phytochemicals (Mohanraj et al., 2018). The second database used, LOTUS, is one of the most

comprehensive and annotated resources for the occurrence of natural products, freely available and unrestricted (Rutz et al., 2022). Finally, the third database was NPASS, which contains over 32,287 organisms and approximately 96,481 natural products (Zeng et al., 2018).
In this research, a compound named E-64 [(L-*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane], which can irreversibly bind to cysteine proteases, was used as a control. The reason was that, in an *in vitro* study, it had potent inhibitory effects on G. *lamblia* cysteine proteases (Carvalho et al., 2014). This choice is further supported by numerous studies demonstrating E-64's efficacy and specificity as a cysteine protease inhibitor. For instance, Sajid and McKerrow highlighted E-64's role as a benchmark inhibitor for papain-like cysteine proteases, emphasizing its usefulness in protease activity assays (Sajid and McKerrow, 2002). Additionally, Caffrey et al. demonstrated E-64's effectiveness in inhibiting cruzain, a major cysteine protease of *Trypanosoma cruzi*, showcasing its broad applicability across parasitic organisms (Caffrey et al., 2001). Moreover, E-64's molecular structure and binding mechanism have been well-characterized through crystallographic studies (Varughese et al., 1989), providing a solid foundation for its use in molecular docking simulations. The compound's stability and irreversible binding properties make it an ideal reference point for comparing the binding affinity and inhibitory potential of novel compounds *in silico*.

2.3. Identification of the active site

The active site of CP2 was detected by the CASTp 3.0 web server to ensure the optimal binding of the compounds. For a ligand to treat a desired disease, the compound should effectively bind to the target protein. CASTp predicts specific amino acid positions at the surface of proteins through matching with SwissProt and the online matching method of Mendelian inheritance in humans (OMIM) (Tian et al., 2018).

2.4. Molecular docking analysis

The binding position of 28 different flavonoids of marjoram on *G. lamblia* CP2 was determined by a molecular docking investigation. This method is a part of computer-aided drug design. In the current study, we used PyRx software as a virtual screening tool for ligands. This software calculates the binding energy between a specific ligand and its target protein in terms of kcal/mol using AutoDock4 and AutoDock vina software and ranks the binding force of different ligands (Dallakyan and Olson, 2014; Mohammad et al., 2020). After the docking process, ligand-protein interactions were assessed with BIOVIA Discovery Studio Visualizer software.

2.5. Pharmacokinetic studies

In this step, the ADME properties of the flavonoids with the least binding energy with CP2 in the docking study were evaluated. The evaluation of ADME (Absorption, Distribution, Metabolism, and Excretion) properties is essential to confirm whether a molecule is suitable for further research. The reason is that ADME properties fundamentally affect a molecule's pharmacological performance (Yamashita and Hashida, 2004). In the present research, the ADME properties of selected flavonoids were evaluated with the Swiss-ADME online server.

2.6. Toxicity analysis

The safety of selected marjoram flavonoids was determined using the admetSAR2 and ProTox-II online servers. The admetSAR 2.0 online server evaluated the selected phytochemicals for possible carcinogenicity, mutagenicity, immunotoxicity, and deleterious effects on signaling pathways (Raies and Bajic, 2016). In addition, further toxicity studies were conducted using the ProTox-II server. This server examines the toxic effects of a compound on different toxicological pathways, such as nuclear receptor signaling pathways and stress response pathways (Banerjee et al., 2018).

2.7. Molecular dynamics (MD) simulation

The GROMACS 5.1.4 software and the GROMOS 96 43a1 force field were employed to identify intermolecular interactions (Van Der Spoel et al., 2005). The molecular environment was defined as separate simulation cubic boxes with a pH of 7 to match the pKa value. We chose an orthorhombic periodic boundary box shape for

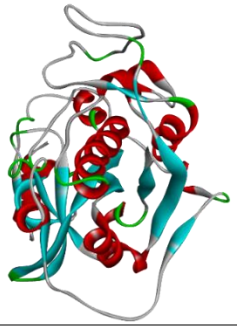
both sides of the boxes, with a distance of 10 Å to maintain a specific volume. Counter ions were added to neutralize the charge of the complexes. The entire system underwent a minimization process using the steepest descent method with 400 steps. Electrostatic interactions were calculated using the particle mesh Ewald method. The simulations were conducted at a temperature of 300 K for 100 nanoseconds.
The stabilized structure obtained from the system's trajectory was used to consider the quality of protein geometry and structure folding reliability. The simulation outputs were used to extract different molecular behaviors of both complex structures. These include the number of hydrogen bonds and the root-mean-square deviation (RMSD).

3. Results and Discussion

3.1. Prediction and confirmation of the three-dimensional structure of cysteine proteases

The 3D model structure of CP2 was predicted using the SWISS-MODEL web server. The best-predicted model with the highest GMQE (Global Model Quality Estimation) was saved. Table 1 shows the three-dimensional structure and properties of CP2. Furthermore, the stereochemical quality of the model was evaluated with PROCHECK analysis, and the significant presence of amino acids in the desired regions indicated good spatial characteristics of the designed model. Fig. 1 shows the position of amino acids in the Ramachandran diagram. The percentage of amino acids in favorable, allowed, semi-allowed, and non-allowed regions of the CP2 was (89.9, 8.4, 0.8, and 0.8 (Fig. 1).

Table 1
Three-dimensional structure and characteristics of CP2 of Giardia.

Protein Name	UniProt ID	Proposed functions	Modeled 3D structure
CP2 (giardipain-1)	A8BTG7	Excystation, induction of apoptosis, disruption of tight junctions, Encystation, degradation of internalized proteins, degradation of tight junction proteins and host chemokines	

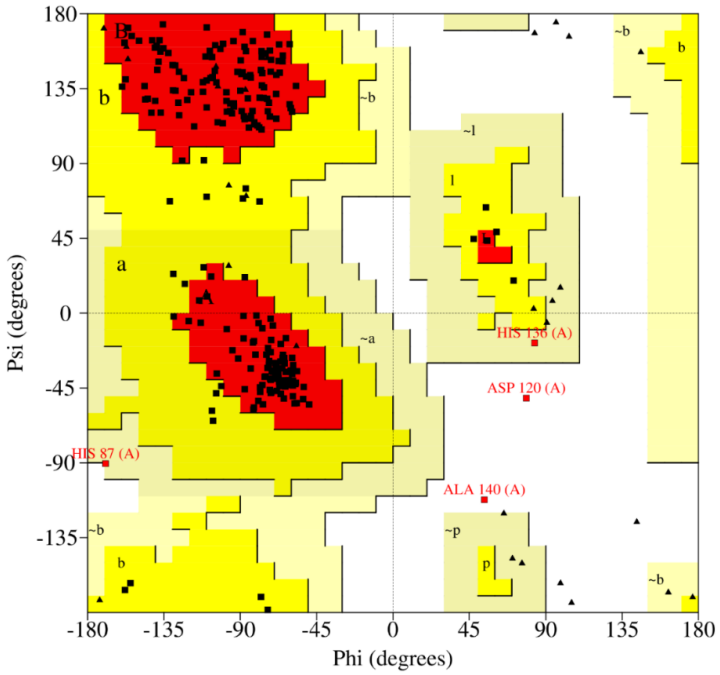


Fig. 1. Ramachandran diagram of the structure of CP2.

In the next step, the compatibility between the CP2 model and its sequence was assessed using VERIFY 3D software. A positive compatibility score on the VERIFY 3D chart indicates acceptable side-chain environments. In the structural model in our study, all amino acids received scores above zero. This indicates a high level of agreement with the experimental structures. Moreover, the ERRAT server was used to analyze non-bonded interactions between atoms. The results showed an overall quality factor of 99.17, which is an acceptable score. Finally, the Z-score obtained through the PROSA web server evaluated the total energy changes in the structural models. This score focuses on the energy distribution of random conformations derived from natural proteins and serves as a measure of the overall quality of the structural model. PROSA analysis showed that the predicted structural model has a high level of agreement with laboratory-derived structures and is comparable in accuracy to natural structures. Table 2 shows the quality assessment of the predicted structure models with VERIFY 3D, PROCHECK, ERRAT, and PROSA tools.

Table 2
Validation results of modeled structure.

Protein	**Server**			
	ERRAT	VERIFY 3D	PROCHECK	PROSA
CP2	99.17	94.01	98.3	-7.14

3.2. Ligand obtaining and preparation

The marjoram plant flavonoids were found in NPASS, LOTUS, and IMPPAT databases. Then, the plant flavonoids were searched in the PubChem database, and their structures were saved in SDF format. The structure and characteristics of these flavonoids are given in Supplementary Table 1. In the last step, proteins and ligands were converted to pdbqt format using the Autodock tool for molecular docking.

3.3. Prediction of active site position

A sequence of amino acids forms the active site of a protein where a ligand temporarily binds to its target protein. The interaction between an active site and a ligand may lead to the formation of a strong bond between them. Therefore, predicting an active site in a protein is important in molecular docking studies. The results of active site prediction are shown in Fig. 2.

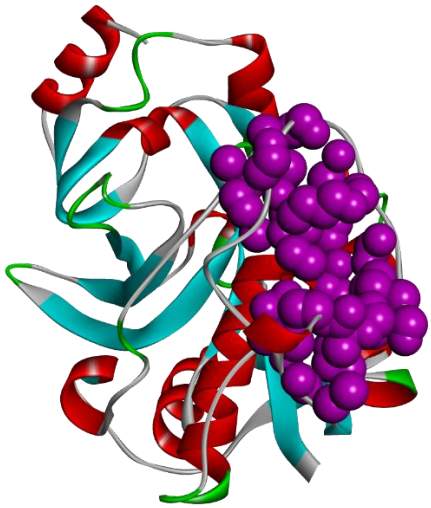


Fig. 2. Active sites of *G. lamblia* CP2 protein.

3.4. Molecular docking

In drug discovery, molecular docking is an excellent method for understanding the interactions between a ligand and a receptor and predicting the structure of receptor-ligand complexes. This method also examines the tendency of a ligand to bind to a receptor. The PyRx software package was used to conduct molecular docking in the study. A part of this package is AutoDock Vina, which performs molecular docking. The software determined the best intermolecular framework between various cysteine proteases and 28 flavonoids of marjoram. The results showed that the binding affinity of the flavonoids varies between -6 and -8.9 kcal/mol.

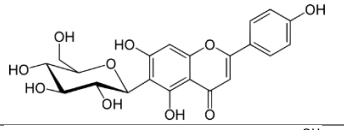
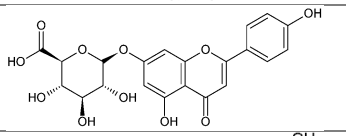
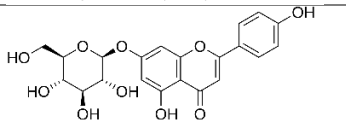
Moreover, among 28 different flavonoids, the top three compounds were selected based on having the highest affinity with cysteine proteases. Table 3 shows the binding affinity between the flavonoids and CP2 protein. The structure and general information of marjoram flavonoids are depicted in Table 4. Table 2 in the Supplementary Materials presents the molecular docking scores of additional flavonoids when interacting with the CP2 protein.

Table 3

Molecular docking results of three marjoram flavonoids that had the highest binding affinity with CP2 protein compared with the control compound (E64).

Protein	**Ligand docking score**			
	isovitexin (kcal/mol)	apigenin 7-*O*-Methylglucuronide (kcal/mol)	cosmosiin (kcal/mol)	E64 (kcal/mol) Control
CP2	-8.4	-8.7	-8.9	-5.9

Table 4
Characteristics of three marjoram flavonoids that had the highest binding affinity.

Flavonoids	PubChem ID	Chemical formula	2D structure
Isovitexin	162350	$C_{21}H_{20}O_{10}$	
Apigenin 7-O-Methylglucuronide	13844658	$C_{22}H_{20}O_{11}$	
Cosmosiin	5280704	$C_{21}H_{20}O_{10}$	

3.5. Protein-ligand interaction analysis

The interactions between three selected ligands and five cysteine proteases were investigated using the BIOVIA Discovery Studio Visualizer. This software provides tools for visualization and analysis of complex molecular structures, enabling a detailed analysis of interactions between ligands and proteins. Fig. 3 shows the interactions between the top three flavonoids and the *G. lamblia* CP2 protein.

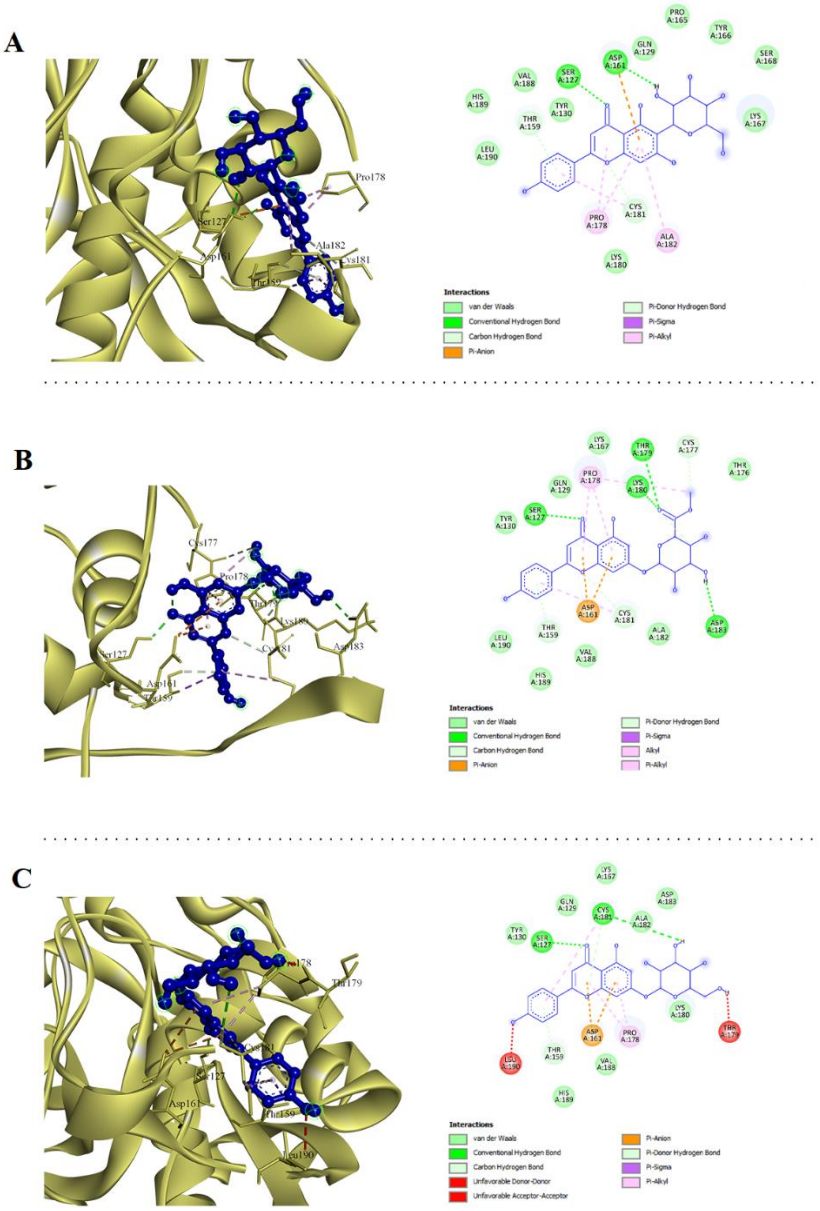


Fig. 3. Interactions between flavonoids and Giardia CP2 protein. (A) Interaction with isovitexin, (B) Interaction with apigenin 7-*O*-Methylglucuronide, (C) Interaction with cosmosiin.

Table 5
List of binding interactions between marjoram flavonoids with CP2 protein.

Compound	Amino Acid	Distance (Angstroms)	Bond Type	Bond Description
E64 (Control)	ASP161	4/57332	Electrostatic	Attractive Charge
	THR159	2/21723	Hydrogen	Hydrogen Bond
	ASP161	2/16399	Hydrogen	Hydrogen Bond
	ASP161	2/24828	Hydrogen	Hydrogen Bond
	LYS167	5/05534	Hydrophobic	Alkyl
	PRO178	3/72483	Hydrophobic	Alkyl
Isovitexin	SER127	2/00619	Hydrogen	Hydrogen Bond
	ASP16	2/66125	Hydrogen	Hydrogen Bond
	CYS181	3/44171	Hydrogen	Carbon-Hydrogen Bond
	ASP161	3/68491	Electrostatic	Pi-Anion
	THR159	3/00865	Hydrogen	Pi-Donor Hydrogen Bond
	THR159	3/63319	Hydrophobic	Pi-Sigma
	PRO178	5/07647	Hydrophobic	Pi-Alkyl
	ALA182	5/22877	Hydrophobic	Pi-Alkyl
	PRO178	4/24999	Hydrophobic	Pi-Alkyl
	CYS181	5/13527	Hydrophobic	Pi-Alkyl
Apigenin 7-*O*-methylglucuronide	SER127	2/33341	Hydrogen	Hydrogen Bond
	THR179	2/59538	Hydrogen	Hydrogen Bond
	LYS180	2/46829	Hydrogen	Hydrogen Bond
	ASP183	2/46838	Hydrogen	Hydrogen Bond
	CYS181	3/47747	Hydrogen	Carbon-Hydrogen Bond
	LYS180	3/52097	Hydrogen	Carbon-Hydrogen Bond
	CYS177	3/53901	Hydrogen	Carbon-Hydrogen Bond
	ASP161	4/39519	Electrostatic	Pi-Anion
	ASP161	4/1782	Electrostatic	Pi-Anion
	THR159	2/93609	Hydrogen	Pi-Donor Hydrogen Bond
	THR159	3/85346	Hydrophobic	Pi-Sigma
	PRO178	4/64077	Hydrophobic	Pi-Alkyl
	PRO178	4/06483	Hydrophobic	Pi-Alkyl
	CYS181	5/00798	Hydrophobic	Pi-Alkyl
Cosmosiin	SER127	2/11811	Hydrogen	Conventional Hydrogen Bond
	CYS181	2/83215	Hydrogen	Conventional Hydrogen Bond
	CYS181	3/42816	Hydrogen	Carbon Hydrogen Bond
	ASP161	4/33687	Electrostatic	Pi-Anion
	ASP161	4/19939	Electrostatic	Pi-Anion
	THR159	2/9324	Hydrogen	Pi-Donor Hydrogen Bond
	THR159	3/7358	Hydrophobic	Pi-Sigma
	PRO178	4/64077	Hydrophobic	Pi-Alkyl
	PRO178	4/06483	Hydrophobic	Pi-Alkyl

	CYS181	5/00798	Hydrophobic	Pi-Alkyl

3.6. Pharmacokinetic study

The three flavonoids having the highest binding energy with the target CP2 protein in the previous step (Isovitexin, apigenin 7-*O*-methylglucuronide, and cosmosiin) were selected for pharmacokinetic studies. In this stage, using the SwissADME online server, the ADME properties of the chosen marjoram flavonoids were determined. Table 6 shows the physicochemical characteristics and pharmacokinetic properties of these flavonoids.

Table 6
Pharmacokinetic characteristics of three marjoram flavonoids that had the highest binding affinity.

	Flavonoids		
Properties	Isovitexin	Apigenin 7-*O*-methylglucuronide	Cosmosiin
Molecular weight	432.4	460.39	432.38
Heavy atoms	31	33	31
Aromatic heavy atoms	16	16	16
Rotatable bonds	3	5	4
Hydrogen bond donors	10	11	10
Hydrogen bond acceptors	7	5	6
Lipophilicity (Log Po/w)	1.94	3.39	2.17
Water solubility (Log S (ESOL))	Soluble	Soluble	Soluble
Oral bioavailability	Low	Low	Low
Synthetic accessibility	4.99	5.19	5.12
Blood-brain barrier penetration	No	No	No
Lipinski rule of five	Passes	Passes	Passes
Ghose	Passes	Passes	Passes
Pfizer rule	Passes	Passes	Passes
Golden Triangle	Passes	Passes	Passes

3.7. Toxicity tests

Toxicity assessment is a crucial step in computational drug design to determine the safety of potential drug candidates. We assessed the toxicity of the selected flavonoids using the admetSAR and ProTox-II online servers. The admetSAR server predicts multiple toxicity endpoints, such as hERG inhibition, AMES mutagenicity, carcinogenicity, and androgen receptor activity. Meanwhile, the ProTox-II server evaluates additional toxicity parameters like hepatotoxicity, immunotoxicity, general cytotoxicity, and nuclear receptor signaling. Table 7 shows the results of toxicity tests for the selected compounds. Our toxicity analysis revealed that apigenin 7-*O*-methylglucuronide exhibited no significant toxicity across the different assays.

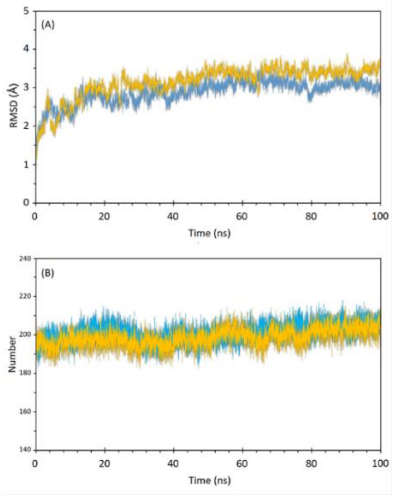
Table 7
Toxicity test results for three selected flavonoids.

Properties	**Flavonoids**		
	Isovitexin	Apigenin 7-O-methylglucuronide	Cosmosiin
Carcinogenicity	-	-	-
Eye damage	-	-	-
Ames mutagenicity	+	-	+
Renal toxicity	-	-	-
Cellular toxicity	-	-	-
hERG blocker	-	-	-
Hepatic toxicity	-	-	-
Respiratory toxicity	-	-	-
Skin sensitization	-	-	-
Androgen receptor (AR)	-	-	-
Aromatase	-	-	-
Estrogen receptor alpha (ER)	-	-	-
Acute oral toxicity in mice	-	-	-
FDAMDD	-	-	-

3.8. Molecular dynamics simulation

Molecular dynamics (MD) simulation was employed to evaluate the stability of protein-ligand complexes, involving the execution of simulations for a period of 100 nanoseconds. The aim was to analyze the steady nature and stability of the complexes by comparing them to a reference antagonist that binds to all target proteins. The results were presented in a root mean square deviation (RMSD) plot, showing that the Giardia CP2 protein with apigenin 7-*O*-methylglucuronide and control compound complexes were stable during the simulation. Furthermore, the average number of protein-ligand intermolecular hydrogen bonds of Interaction between apigenin 7-*O*-Methylglucuronide and Giardia CP2 protein is generally higher than interaction with the control compound structure. This observation reveals apigenin 7-*O*-methylglucuronide and Giardia CP2 protein's higher stability than the control complex structure, as represented in Fig. 4.

Fig 4. (A) The RMSD plot for the complex of Giardia CP2 protein with apigenin 7-*O*-Methylglucuronide (blue) and Giardia CP2 protein with E64 (orange) and (B) the average number of protein-ligand intermolecular hydrogen bonds in the complex of Giardia CP2 protein with apigenin 7-*O*-Methylglucuronide (blue) and Giardia CP2 protein with E64 (orange).



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3.9. Interpretation of results

The present research showed that the flavonoids in the marjoram plant could bind to the active site of CP2 (Giardipain-1) enzyme secreted by the parasite. CP2 plays a major role in the invasion of the unicellular organism to the epithelial surfaces by breaking down extracellular matrix proteins such as laminin and fibronectin. The enzyme also destroys the tight junctions between epithelial cells, disrupting the integrity of the intestinal epithelium. Furthermore, CP2 helps the parasite feed by breaking down mucin and immunoglobulins. In addition to these functions, CP2 assists in immune evasion by degrading secretory immunoglobulins and defensins. By destroying the proteins of the microvilli of the intestinal lining cells (such as villin), CP2 also interferes with the absorption of nutrients The present research showed that the flavonoids in the marjoram plant could bind to the active site of the CP2 (Giardipain-1) enzyme secreted by the parasite. CP2 plays a significant role in the invasion of unicellular organisms to the epithelial surfaces by breaking down extracellular matrix proteins such as laminin and fibronectin. The enzyme also destroys the tight junctions between epithelial cells, disrupting the intestinal epithelium's integrity. Furthermore, CP2 helps the parasite feed by breaking down mucin and immunoglobulins. In addition to these functions, CP2 assists in immune evasion by degrading secretory immunoglobulins and defensins. By destroying the proteins of the microvilli of the intestinal lining cells (such as villin), CP2 also interferes with the absorption of nutrients (Ortega-Pierres et al., 2018; Liu et al., 2019; Quezada-Lázaro et al., 2022).

In line with our results, an in vivo study by Dawoodi et al. has shown that the plant extract has anti-giardia properties (Davoodi and Abbasi-Maleki, 2018). In addition, some studies show the antiprotozoal effects of plant flavonoids (Hernández-Bolio et al., 2015; Ticona et al., 2022). Therefore, at least part of the anti-Giardia effects of the marjoram plant can be attributed to the targeting of Giardia CP2 by its flavonoids. The results of the present study showed that the flavonoids isovitexin, cosmosiin, and apigenin 7-O-methylglucuronide have the highest binding affinity with the active site of CP2. An in-depth analysis of the binding interactions showed that the flavonoids are highly hydrophobic, and hydrogen bonding is established between the compounds and CP2 (Fig. 3).

The Pharmacokinetic parameters of a drug usually determine its behavior in the human body based on its ADME (absorption, distribution, metabolism, and excretion) properties. Evaluating these parameters during the drug design process is significant in the success of a drug in clinical trials. The permeability of a drug-like molecule across the biological barrier is a leading factor in Pharmacokinetics. Molecular weight and the topological polar surface area (TPSA) influence this property. Higher molecular weight decreases the drug permeability, while lower TPSA indicates higher drug permeability (Yang et al., 2017). Absorption of drug molecules in the human body is influenced by lipophilicity and is calculated by the logarithm of the mineral and water phase partition coefficient of the target molecule (LogP). When the parameter is high in a candidate drug, it indicates a lower absorption and vice versa. The solubility of a drug in water is measured with the LogS parameter, and a lower Log S indicates a higher solubility of a molecule. The number of hydrogen bond donors and acceptors can also affect the capacity of a drug molecule to pass through biological membranes. Because all the selected compounds have low intestinal absorption, the possibility of oral toxicity is reduced with the consumption of these flavonoids. On the other hand, *G. lamblia* resides in the intestinal lumen, making the parasite a suitable target for these flavonoids. Table 6 shows the pharmacokinetic properties of the selected marjoram flavonoids.

The toxicity of a drug candidate is evaluated to avoid potential harm to target organisms. Computational methods for toxicity assessment save time and money compared to animal and cellular toxicity tests. The toxicity of flavonoids screened by the admetSAR online server indicates that the compounds do not have carcinogenic properties. The toxicity tests also showed the non-inhibitory effect of flavonoids on hERG toxicity, which is crucial as inhibition of the hERG gene can cause fatal cardiac arrhythmia. Although all three flavonoids had no toxicity in most toxicity tests, isovitexin and cosmosiin showed toxicity in the Ames mutagenesis evaluation. This evaluation is widely used in toxicity studies. Although it does not directly

measure carcinogenicity, it provides a rapid and cost-effective way to assess the mutagenic potential of chemicals (Claxton et al., 2010).
The stability of the flavonoid-CP2 complexes was evaluated by assessing van der Waals, polar, and non-polar bonds. It was shown that hydrogen bonds play a significant role in the binding of flavonoids with CP2. Comparing the binding affinity of the selected flavonoids and the control drug (E64) with CP2's active sites, we found that the flavonoids have a higher affinity for the enzyme. In a previous *in vitro* study, E64 has shown an inhibitory effect on Giardia cysteine proteases. Therefore, the final screened flavonoid in our research (apigenin 7-*O*-methylglucuronide) may have inhibitory activity against Giardia CP2. Finally, the results of molecular dynamic simulation confirm the stability of the apigenin 7-*O*-methylglucuronide and Giardia CP2 complex.

Concluding remarks

Giardiasis is a parasitic disease with a global distribution caused by *Giardia lamblia*. Common drugs used to treat this disease have many side effects that limit their use. Nowadays, molecular docking techniques have accelerated the discovery of new medicines of plant origin. Plant flavonoids have shown antiparasitic properties. In the current investigation, the screening of flavonoids derived from *O. vulgare* utilizing this method indicated that apigenin 7-*O*-methylglucuronide might have inhibitory effects against giardipain-1 (SP2), which is an important virulence factor of this parasite. Molecular dynamics results also confirmed the molecular docking data. Therefore, this flavonoid may play a role in treating this disease as a potential drug. However, to introduce this flavonoid as a drug against this infection, it is essential to undertake *in vitro* studies, along with supplementary research involving animal models and clinical trials.

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Author contribution statement

Conceptualization and literature search were performed by Amir Abbas Barzegari and Sepideh Parvizpour. The first draft of the manuscript was prepared by Milad Zare. Sepideh Parvizpour and Amir Abbas Barzegari critically analyzed and gave suggestions to finalize the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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