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Original article

In vitro leishmanicidal potential and silico study of flavonoids isolated from *Pistacia integerrima* Steud ex Brandis



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ARTICLE INFO

Article history:

Received 18 July 2022

Revised 4 January 2023

Accepted 18 January 2023

Available online 24 January 2023

Keywords:

Pistacia integerrima

Anacardiaceae

Flavonoids

Leishmanicidal activity

Docking studies

ABSTRACT

Objective: Medicinal plants are the mainstay of various chemical constituents responsible for curing or mitigating different pathological and non-pathological health issues. In this regard, the current research work was subjected to finding the best effective, new and safe leishmanicidal molecules. *Pistacia integerrima* is an important medicinal plant growing in Asia with various ethnopharmacological uses such as skin, respiratory and hepatic disorders. The folklore of this plant in skin problems encouraged us to evaluate the isolated compounds for leishmanicidal activity.

Methods: In the current study, whole ethyl acetate extract was subjected to chromatographic analysis affording two flavonoid constituents, namely naringenin (**1**) and 3,5,4'-trihydroxy-7-methoxy-flavanone (**2**). The extract and isolated constituents were tested for leishmanicidal activity. Both compounds were docked into the target binding site (PDB ID: 5HJ9) using MOE 2016. Arginase was taken as the targeted enzyme which is an important enzyme for leishmania species' survival. Arginase up-regulates amastin that impacts amastigote replication and survival. This approach aimed to explore the binding modes of the two isolated flavonoids and to predict their inhibitory activity against arginase.

Results: The two flavonoid compounds isolated from *P. integerrima* were evaluated for their anti-leishmanial activity. Compounds **1** and **2** were tested at a concentration of 5 µg/mL against the selected Leishmanial species. The percent inhibition was 70.43 and 74.76 regarding compounds **1** and **2**, respectively with IC₅₀ of 29.43 and 16.43 µM. The reference standard drug used, amphotericin-B, displayed the highest leishmanicidal effect (IC₅₀ of 0.22 µM) with 84.09 % inhibition at half concentration compared to compounds **1** and **2**. The molecular docking studies of these two compounds and the standard drug were performed in MOE software. According to the findings, compound **1**, naringenin, docked well in the binding site, with a docking score of -9.28 followed by compound **2**, 3,5,4'-trihydroxy-7-methoxy-flavanone with a docking score of -8.37.

Conclusion: Both of the tested samples demonstrated excellent leishmanicidal effects. This pharmacological inhibitory profile provides a strong scientific profile to the folklore of *Pistacia integerrima* in the treatment of skin disorders especially leishmanial. A further mechanistic study is recommended to find the extract target responsible for the said activity.

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Peer review under responsibility of King Saud University.



1. Introduction

Plants are the key figures for the life of human beings in various aspects. They are important for all needs of human beings in terms of clothing, shelter, food, flavors, fragrances, and especially medicine. Medicinal herbs have designed the foundations of traditional

<https://doi.org/10.1016/j.jksus.2023.102572>

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medicinal systems, including Chinese medicine, Ayurveda, and Unani (Gurib-Fakim, 2006; Naz, 2022). The practice of this medicinal plant-based therapy is found in all corners of the world in various shapes and they are considered safe and free of toxic effects by the world population. This concept of being free from adverse effects increased many folds the consumption of these plants for the treatment or mitigation of various diseases. Natural products are effective due to their secondary metabolites. A lot of secondary metabolites have been isolated and even in practice from these plants. These traditional systems have led to the discovery of key drugs like the well know central analgesic morphine and the best hepatoprotective drug silymarin. In the current modern era, human beings are facing a lot of health problems and still wait for the discovery of new natural products for the treatment of a lot of diseases. Search for novel drugs is a permanent challenge. Phytochemists first selected medicinal plants based on popular usage (Gurib-Fakim, 2006). About 5.2 billion peoples live in less developed countries and WHO has estimated that 80 % of these countries depend on traditional medicine for their primary health-care (Davidson-Hunt, 2009). Herbal products are the backbone of traditional medicine and nearly 3.3 billion people regularly use herbal products (Liu et al., 2009; Ahvazi et al., 2012). Medicinal plants are the key sources of secondary metabolites and their biological potential is due to the presence of these bioactive compounds (Anand et al., 2019). The medicinal plant is a rich source of several bioactive compounds like Pistagremic acid (Ullah et al., 2014), Diospyrin (Ullah et al., 2015a), and 8-hydroxyisodiospyrin which possess excellent biological potential (Ullah et al., 2015b).

Pistacia integerrima (zebrawood) is a member of the Anacardiaceae family native to Asia (Main et al., 2001; Patel et al., 2018). *P. integerrima* is a deciduous tree used in traditional folk medicine for curing various diseases such as phthisis, cough, vomiting, diarrhea, jaundice, asthma, chronic wounds, and dysentery, and employed as an antiseptic (Chopra et al., 1986; Khan et al., 2004). In various countries, zebra wood galls are used in herbal formulations for the treatment of diarrhea and dysentery. Aqueous extracts of that plant were found effective in the curing of hepatic injuries (Ansari et al., 1996; Ansari et al., 1993). Gall extracts of *P. integerrima* (Fig. 1) were also reported to have promising analgesic, hypouricemic, antioxidant, and anti-inflammatory activities (Ahmad et al., 2006; Ahmad et al., 2008). The crude extracts and isolated *P. integerrima* have been documented for potential anticancer, antioxidant, and xanthine oxidase inhibitory effects (Bibi et al., 2012; Ahmad et al., 2010). Various classes of bioactive secondary metabolites such as terpenoids, sterols, flavonoids, phenolic compounds, and dihydromalvalic acid have been isolated from the different parts of *P. integerrima* (Zahoor et al., 2018; Joshi et al., 2010; Ahmad et al., 2020). These secondary metabolites have

been reported to show anticancer, analgesic, antioxidant, and enzyme-inhibitory activities (Rauf et al., 2017a, 2017b, 2017c; Rauf et al., 2017a). Various class of compounds such as triterpenes and flavonoids has been isolated from various part of *Pistacia integerrima* which are reported as anticancer (Rauf et al., 2017b), muscle relaxant (Rauf et al., 2018; Rauf and Patel, 2017), analgesic, phosphodiesterase, glucosidase activities (Alhumaydhi et al., 2021; Bawazeer et al., 2021). The phytochemicals isolated from galls of *Pistacia integerrima* also exhibited excellent antimicrobial and α -glycosidase activity. Hemeg et al., 2022a; Hemeg et al., 2022b). Here, we report on the *in vitro* leishmanicidal potential of *P. integerrima* ethyl acetate extracts/compounds and the *in silico* study of two flavonoids **1** and **2**.

2. Materials and methods

2.1. Plant collection

Galls of *Pistacia integerrima* were collected in May 2021, from Tehsil Khall District Dir Lower, KP, Pakistan. The galls were selected at maturity. The collected specimen was brought from the Department of Botany University of Swabi, KPK, Pakistan. The identification of the specimen was by Dr. Muhammad Ilyas Department of Botany, University of Swabi, and the voucher specimen number No. UOS/Bot-102 was stored at the Botany University of Swabi, KPK, Pakistan.

2.2. Extraction and isolation

The collected galls (7.54 kg) were washed with water to remove the dust part and then cut into small pieces. These small pieces were dried under shade and converted to powder with the help of a grinder machine. The powder plant material was soaked in 50 L methanol and water (80:20) for 18 days according to the published procedure (Rauf et al., 2022a, 2022b, 2022c; Rauf et al., 2017a, 2017b, 2017c). The obtained extract was concentrated in a rotary evaporator under low temperature and pressure to obtain a dry extract (97 g). The dry extract was then dissolved in minimum amount water (200 mL) and fractionated with the help of a separating funnel to respectively non-polar (Hexane) and polar fractions (Chloroform, ethyl acetate). The ethyl acetate fraction (17.1 g) was subjected to glass column chromatography elution being ensured by a chloroform–methanol mixture (92:8, V/V) affording 43 subfractions termed AF hereafter. Based on their TLC profiles, the sub-fractions AF-1 to AF-17 were combined and subjected to repeated column chromatograms (CC) using the same solvent mixture chloroform–methanol (92:8, V/V) yielding two compounds **1** (1.20 g; 99.93 % pure) and **2** (1.02 g; 99.91 % pure).



Fig. 1. Image of *Pistacia integerrima* Steud ex Brandis.

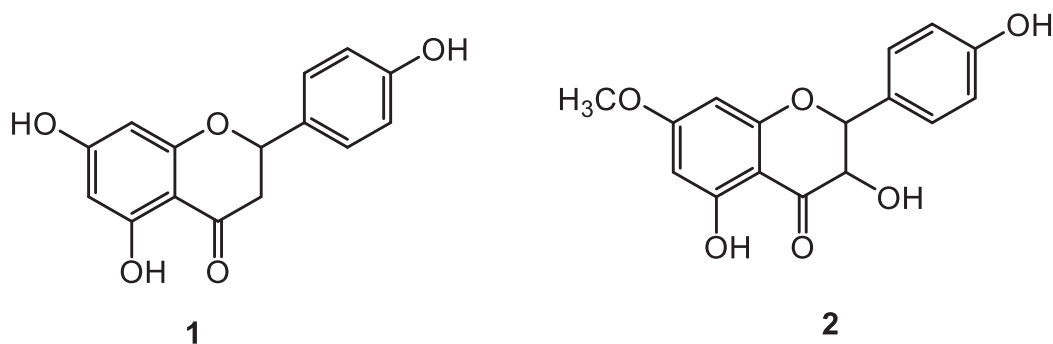


Fig. 2. Chemical structures of the two flavonoid constituents **1** and **2** isolated from *P. integerrima*.

Table 1

Leishmanial activity of flavonoids constituents (**1**, **2**) isolated from *P. integerrima*.

Tested sample	Concentration	Percent effect	IC ₅₀ (μM)
Ethyl acetate Extract	5 μg/mL	64.09 ± 0.80	41.98 ± 1.98
Compound 1	5 μg/mL	70.43 ± 0.70	29.43 ± 0.23
Compound 2	5 μg/mL	74.76 ± 0.43	16.43 ± 0.65
Amphotericin-B	2.5 μg/mL	84.09 ± 0.40	0.22 ± 0.09

Structures of the isolated compounds (**1** & **2**; Fig. 2) were elucidated by comparing their physical and spectroscopic data (FT-IR, ¹H NMR, ¹³C NMR) with those previously reported (Rauf et al., 2015; Rauf et al., 2022a, 2022b, 2022c).

2.3. Leishmanicidal activity

The two isolated compounds were tested for their leishmanicidal activity as per the standard procedure described in the literature (Shah et al., 2019). The two isolated flavonoids were tested against leishmanial spp I, e L. major. The selected species were cultured at 25 ± 2 °C on the RPMI-1640 medium following literature recommendations (Shah et al., 2018). Ten percent heated-inactivated FBS was added to the medium. The culture was centrifuged (2000 rpm) out at the logarithmic growth phase of the promastigote and washed with saline. The culture was diluted with fresh medium (up to 10⁶ cells/mL). The procedure was performed in 96-well microplates. The first row was supplemented with 180 mL of the medium while the rest of the wells were filled with 100 mL. The isolated flavonoids (**1** and **2**) were diluted in 5 mL DMSO and added to the medium and the pathogen culture was added to all wells. The negative control well was filled with DMSO and the positive control well was filled with the reference antileishmanial drug used in this study, amphotericin-B. The above-filled microplates were properly handled and inoculated for 72 h. After this time, the leishmanicidal potential was counted. The experiment was repeated three times and the inhibition percentage was calculated as per the reported methods (Shah et al., 2018).

Table 2

Docking analysis of the two flavonoids constituents **1** and **2** isolated from *P. integerrima*.

Compound	Interacting residues	Interaction type	Docking score	E (kcal/mol)
Flavonoid 1	ASP 194 HIS 154	H-donor	−7.32	−2.7
		pi-pi		−0.0
Flavonoid 2	SER 150 HIS 139	H-donor	−6.96	−2.8
		pi-pi		−0.0
Amphotericin-B	ASP 195 SER 150 ASN 143	H-donor	−9.70	−1.4
		H-acceptor		−1.2
		H-acceptor		−2.0

2.4. Docking studies

The ChemDraw Ultra 8.0 was used to draw the 2D structures of the two isolated flavonoid constituents, naringenin **1**, and the 3,5,4'-trihydroxy-7-methoxy-flavanone **2**. The crystal structure of leishmanial Mexicana arginase PDB ID 5HJ9 was retrieved from the PDB (Protein Data Bank) Hai et al., 2016. Energy minimization was fixed and charges were added by the MOE software. The minimized structure was used to dock the compounds in the active site of the receptor. Furthermore, the best score compounds were selected and visualized in the MOE software for protein–ligand interaction analysis (Malayeri et al., 2017).

3. Results

The two flavonoid compounds isolated from *P. integerrima* were evaluated for their anti-leishmanial activity, as presented in Table 1. Compounds **1** and **2** were tested at a concentration of 5 μg/mL against the selected Leishmanial species. The percent inhibition was 70.43 and 74.76 regarding compounds **1** and **2**, respectively with IC₅₀ of 29.43 and 16.43 μM. The reference standard drug used, amphotericin-B, displayed the highest leishmanicidal effect (IC₅₀ of 0.22 μM) with 84.09 % inhibition at half concentration compared to compounds **1** and **2**.

To investigate the nature of the interactions between the two flavonoids and the targets, both compounds were docked into the target binding site (PDB ID: 5HJ9) using MOE 2016 (Mirzaei et al., 2020). The targeted enzyme was arginase, which is an important enzyme for leishmania species' survival. Arginase up-regulates amastin that impacts amastigote replication and survival. This approach aimed to explore the binding modes of the two isolated flavonoids and to predict their inhibitory activity against arginase. Table 2 shows the overall docking scores and interactions of the tested compounds with arginase. The two flavonoids showed a high binding affinity with arginase though being lower than that of Amphotericin-B. According to the findings, compound **1** docked well in the binding site, with a docking score of −9.28 Fig. 3 (a-c). The oxygen atom of the standard compound forms 2H-bond accep-

tor interactions with Ser 150 and ASN 143 and one H-donor interaction with the Asp 195 residue of the receptor. The O atom of compound 2 forms two H bond donor interactions with ASP 194 and GLU197. Compound 2 forms one H-bond acceptor interactions

and one pi-pi interaction. The O 18 atom of the ligand forms one interaction and 6 ring forms one donor interaction with HIS 139 of the receptor. The interaction images of the two compounds and the reference are shown in Figs. 3-8.

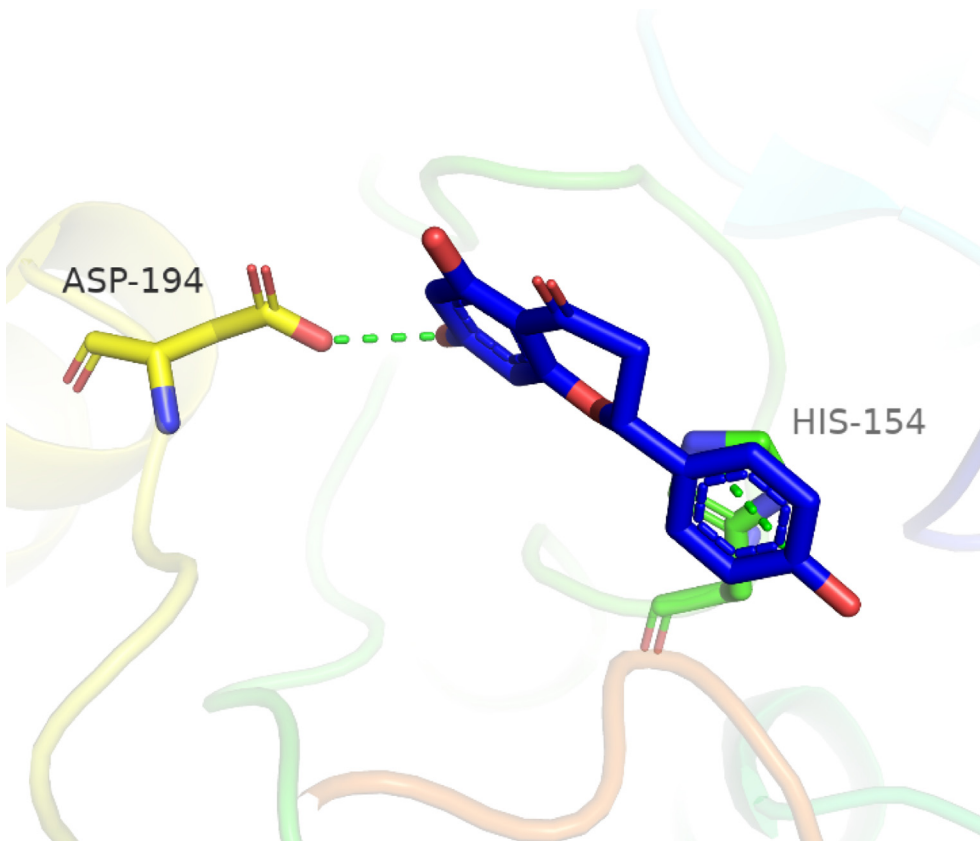


Fig. 3. 3D interaction of flavonoid 1 with active site residues of arginase.

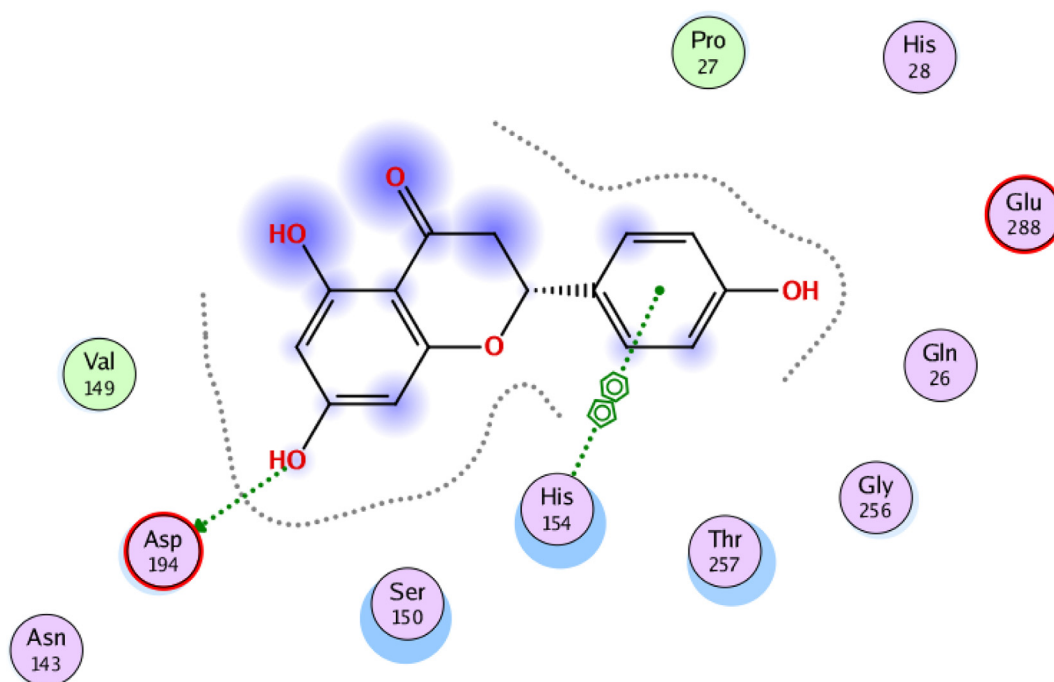


Fig. 4. 2D interaction of flavonoid 1 with active site residues of arginase.

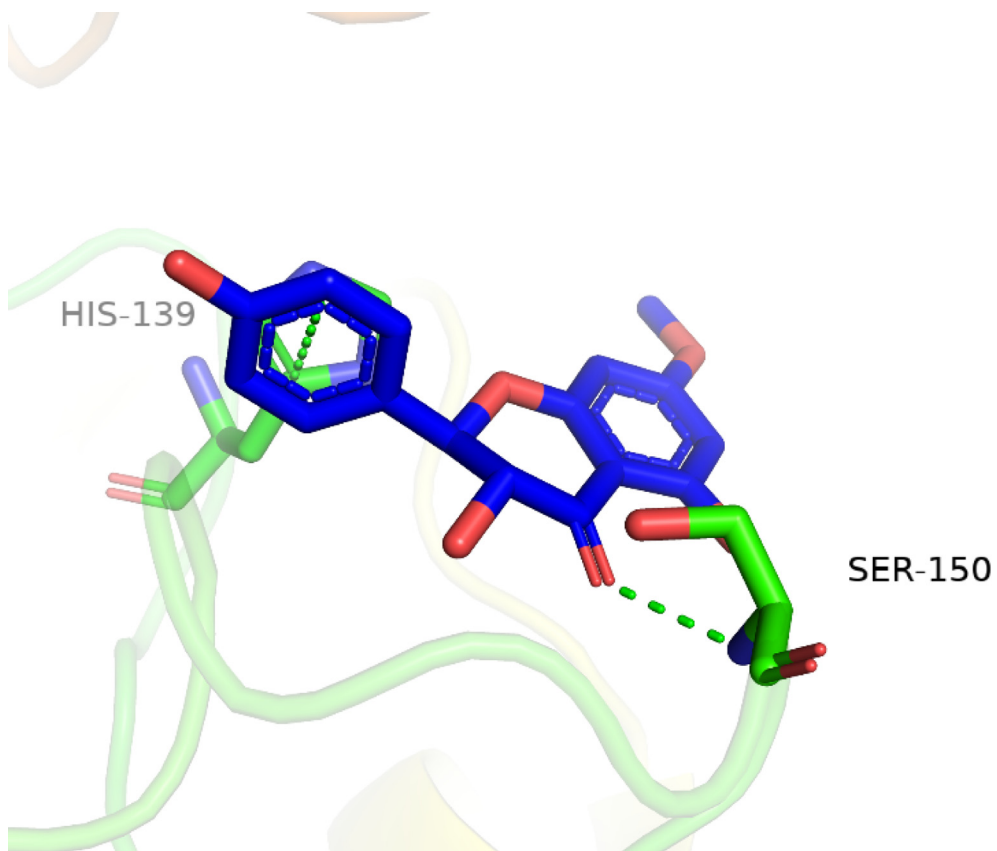


Fig. 5. 3D interaction of flavonoid 2 with active site residues of arginase.

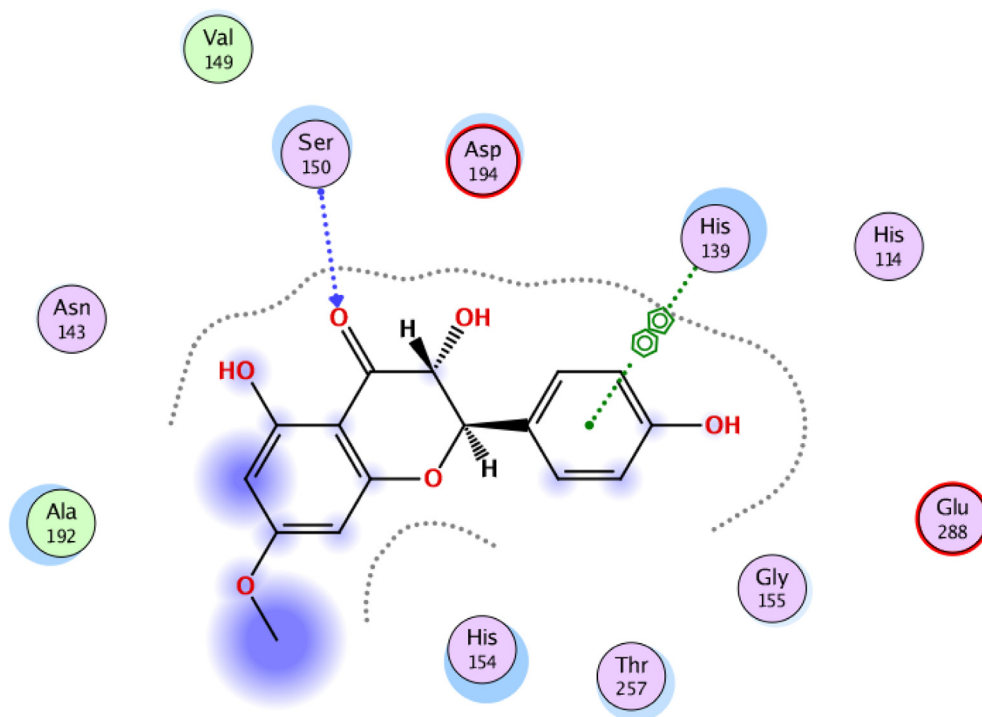


Fig. 6. 3D interaction of flavonoid 2 with active site residues of arginase.

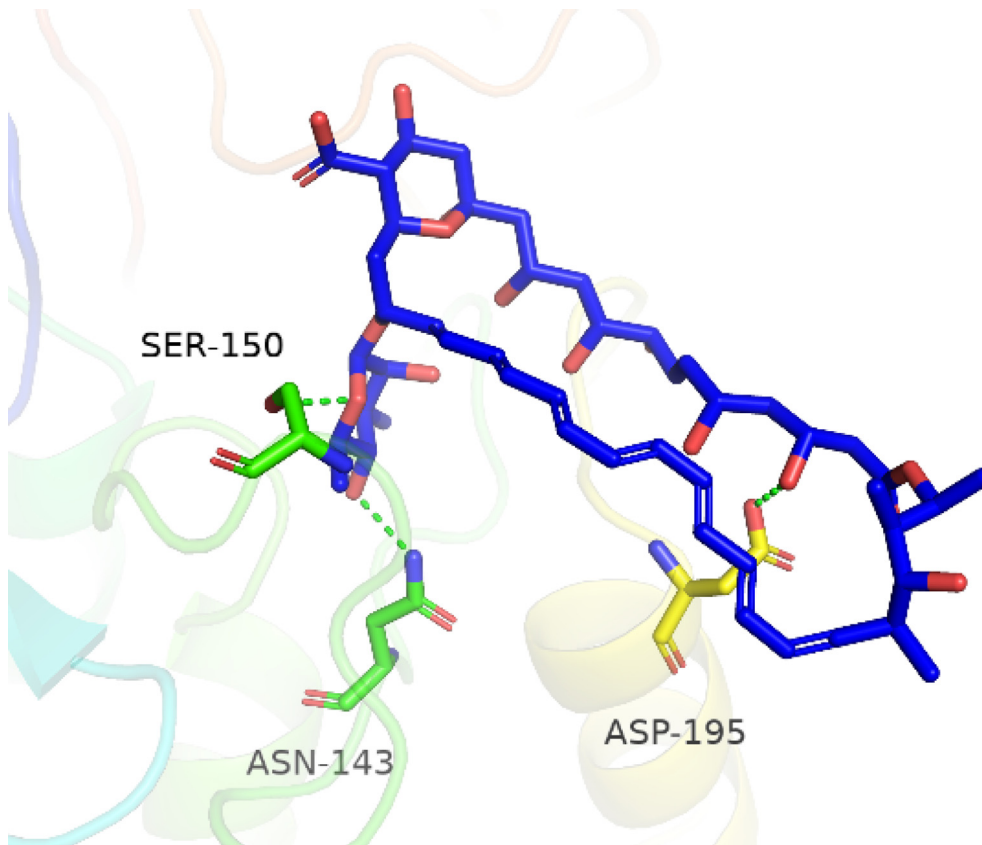


Fig. 7. 3D interaction of Amphotericin-B with active site residues of arginase.

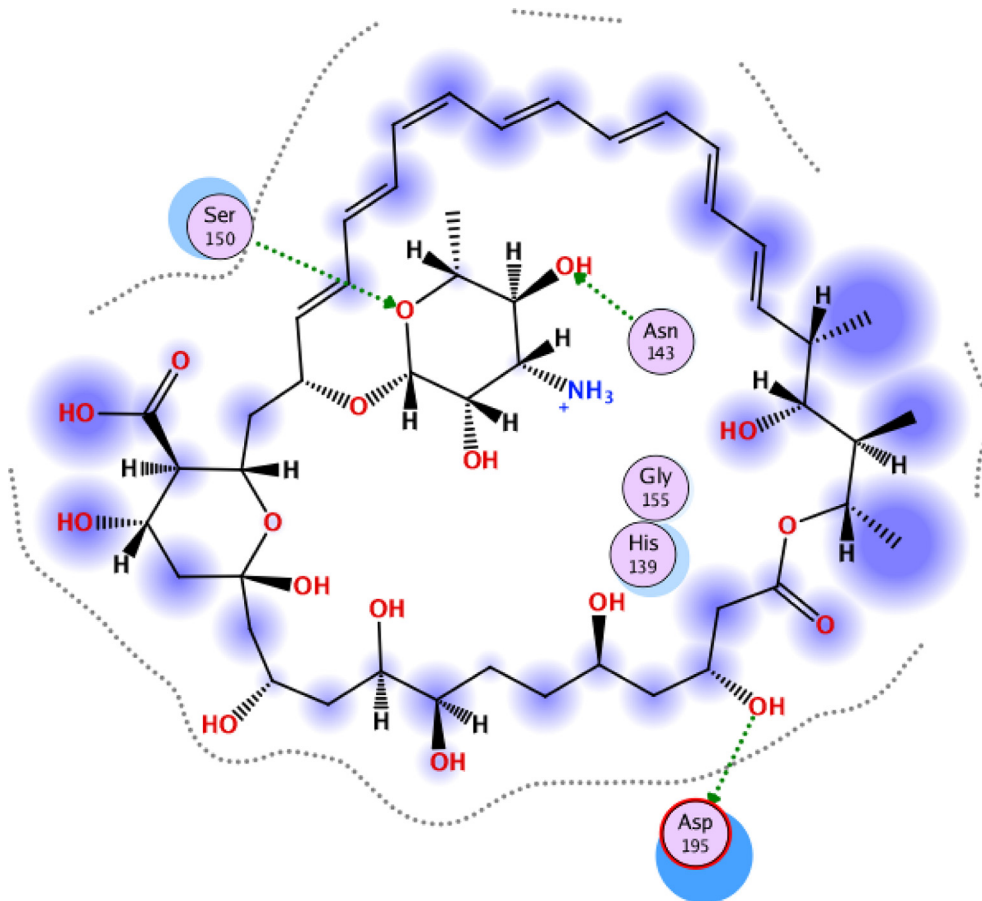


Fig. 8. 3D interaction of Amphotericin-B with active site residues of arginase.

4. Discussion

Leishmaniasis is one of the parasitic diseases which have affected a large population in various countries around the globe (Tuon et al., 2008). This dermatological disorder is considered as a neglected topical disorder (Hotez et al., 2007). Leishmania infection is attributed to leishmanial parasites and is transmitted by the bite of a sandfly (female phlebotomine) (Killick-Kendrick, 1999). The leishmanial infection is also known as black fever which is typically characterized by weight loss, weakness, fever, splenomegaly, and malaise, etc (Nazzaro et al., 2014). The most severe form of leishmaniasis is visceral, commonly called Kala-azar, while the other forms of leishmaniasis are mucocutaneous and cutaneous, the latter being the most common form of leishmaniasis (Sundar, 2002). There are thirty species of leishmania and according to some reports, human leishmaniasis is caused by 21 species out of 30. The multiple species of any pathogens are mostly responsible for drug resistance and failure of treatment. In addition to the resistance problem, poor patient compliance is also one of the major issues with this infection eradication or curing. These problems encouraged researchers to find safe, effective, and economical medicines for the treatment of this infection. Because it is a general perception that the plants based medicine are free of adverse side effects. In addition to free from toxicity, they are also economical. The current study aimed to investigate a natural remedy for the treatment of leishmaniasis as well as to minimize resistance and increase patient compliance. The two flavonoids isolated from *P. integerrima* extract demonstrated significant inhibition of the growth of one leishmanial specie. The percent inhibitory potential of these two compounds may be increased by performing a structure–activity relationship (SAR). The SAR might pave the way for new, effective, and economical remedies such as antileishmanial. These two flavonoids with antileishmanial activities isolated from *P. integerrima* can provide a strong basis for future studies. To investigate the nature of interactions, this study was strengthened by conducting comparative docking studies of the arginase receptor with the two isolated flavonoids and the standard drug. Docking studies demonstrated good qualitative agreement with experimental IC₅₀ values. Both studies concluded that flavonoid **1** interacted more strongly with arginase than flavonoid **2**.

5. Conclusion

The plant *Pistacia integerrima* Steud ex Brandis displays a multi-medicinal potential. The current flavonoids isolated in the present study from that plant, have demonstrated a significant leishmanicidal effect. Further, docking studies of these two compounds and the standard drug used to treat leishmaniasis were performed with the arginase receptor. Docking studies confirmed that the two isolated compounds docked well into the active site of arginase and form strong hydrogen bond interactions with the receptor. So the isolated compounds may possess the potential to inhibit arginase and can be the starting point for potent drugs to treat leishmaniasis.

Data availability

The data connected to this project is given in the text of this paper. The spectroscopic data of naringenin (**1**) and 3, 5,4'-trihydroxy-7-methoxy-flavanone (**2**) is available with contact authors upon request.

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.

Acknowledgment

The authors are thankful to the Researchers Supporting Project number (RSP2023R491), King Saud University, Riyadh, Saudi Arabia.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jksus.2023.102572>.

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