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Research Article

In Vitro and *In Silico* Evaluation of the Antileishmanial Potential of Isolated Compounds from *Ifloga spicata*

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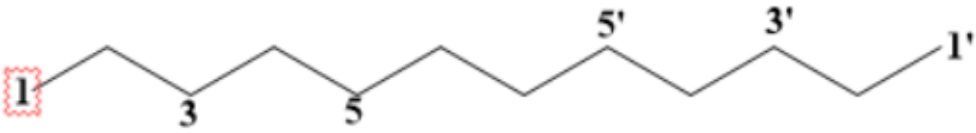
Abstract

floga spicata is a tiny annual herb of the Asteraceae family. It is used to treat a wide range of disorders, including heart diseases, skin diseases, and leishmaniasis. The aim of this research is to evaluate the effectiveness of compounds isolated from *Ifloga spicata* for controlling leishmaniasis, via a combination of *in vitro* and *in silico* studies. *Ifloga spicata* was chosen due to its high concentration of secondary metabolites and its historic use in treating leishmaniasis. According to the increasing sequence of polarity, the crude extract and its derived n-hexane, chloroform, ethyl acetate, methanolic, and aqueous fractions were made. The previously discovered bioactive ethyl acetate soluble fraction was suggested for further investigation using column chromatography. Two chemicals were recovered from the ethyl acetate fraction. The structure of these compounds was determined using Mass, ^{1}H-NMR, and ^{13}C-NMR spectroscopy, which confirmed that the pure compounds were Dodecane and Tetradecane. The compounds were evaluated for antileishmanial activity *in vitro* using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Both the compounds showed promising anti-leishmanial activity. These compounds were subjected to a molecular docking analysis to find their binding energy with the parasite enzyme trypanothione reductase. Both the compounds docked at the optimal position, showing stable binding energies, and chemical bond types. The binding energies of dodecane and tetradecane were -4.5 and -4.7 kcal/mol, respectively. These findings suggest that the secondary metabolites identified from *Ifloga spicata* should be investigated further as natural lead compounds to treat leishmaniasis.

Keywords: *Ifloga spicata*, antileishmanial, molecular docking, structure elucidation, MTT

1. Introduction

The compounds that are produced by plants are referred to as phytochemicals. The phytochemicals are known to possess beneficial health qualities, and they play a key role in reducing the likelihood of developing serious illnesses. Plants are responsible for the all-natural production of these compounds (Jimenez-Garcia et al. 2018). Leishmaniasis is well recognized as a significant global public health concern, especially in Africa, where it causes a substantial number of illnesses and deaths (Chiheb et al. 1999; Hussain et al. 2019; Et-Touys, Fellah, Mniouil, et al. 2016). The condition is attributed to parasites that are spread by phlebotomine insects. There has been a notable rise in research on antileishmanial drugs recently, mostly due to two factors (Bouyahya et al. 2016; Bates 2007): Firstly, the effects of certain treatments, such as antimony compounds, on seeds are both poisonous and costly. In addition, many Leishmania species have shown resistance to synthetic drugs, hence contributing to the development and recurrence of infectious diseases (Aya et al. 2017). These two factors have focused antileishmanial medication development on



IUPAC Name: Dodecane
Molecular formula: C12H26
Molecular weight: 170.33
Physical form: Clear colorless liquid
Boiling Point: 474.16 [K]
Melting Point: 224.5 [K]

Figure 1. Structure and physical properties of compound 1.

screened compounds with selective effectiveness and accessible safety.

Secondary metabolites derived from medicinal plants have many pharmacological features, including antibacterial activity (Fadel et al. 2013) These secondary metabolites are intricate compounds that possess diverse functional structures, including polyphenols, flavonoids, terpenoids, and coumarins. Recent studies have demonstrated the efficacy of medicinal plant products in inhibiting the growth of various Leishmania species, including *L. major* (causing cutaneous leishmaniasis) and *L. infantum* (causing visceral leishmaniasis) (Et-Touys, Fellah, Sebti, et al. 2016). The objective of this study was to examine the antileishmanial characteristics of compounds derived from *I. spicata* mushrooms.

2. Materials and Methods

2.1. Plant Collection and Extraction

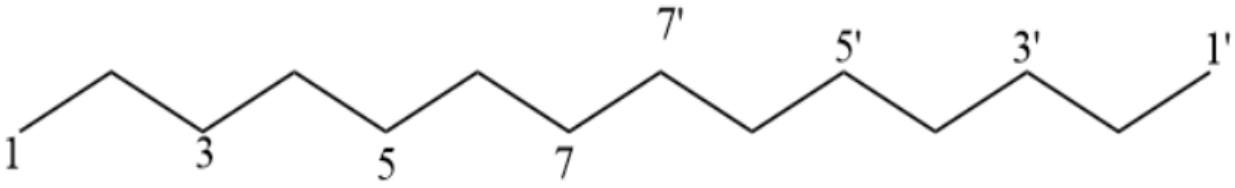
A specimen of *I. spicata* was obtained from the Karak area of Khyber Pakhtunkhwa, Pakistan, in early April 2020. The identification was verified by a plant taxonomist affiliated with the Department of Plant Sciences at Kohat University of Science and Technology. The sample was first washed with tape water, then distilled water, and then shade-dried for 15 days at 45º C. The plant was pulverized using an electric grinder. The powdered sample was soaked in methanol, and the filtrate was evaporated in a rotary evaporator (H. Khan et al. 2013). The methanolic extract produced a 200g greenish-brown semisolid mass.

2.2. Fractionation

The crude extract was diluted in 500ml of water before being fractionated with multiple organic solvents in an increasing polarity sequence (Hussain et al. 2019). Using a separating funnel, five fractions were produced methanolic, chloroform, n-hexane, ethyl acetate, and aqueous. All fractions were stored for future use.

2.3. Compounds Isolation

The ethyl acetate fraction was obtained and then dissolved in a sufficient amount of ethyl acetate to produce a more diluted solution. A small quantity of silica gel was used to adsorb the portion. The column chromatography was filled with the properly dehydrated sample (slurry). The column was flushed with a solvent system consisting of roughly 500 mL of ethyl acetate and n-hexane, in order to increase the polarity of the series. After each run of column chromatography, the solution was concentrated using a rotary evaporator and then stored in a vial. A total of 180 vials were collected. Thin layer chromatography (TLC) was used to detect pure compounds using UV light of several wavelengths, including visible, long, and short wavelengths. The vials containing identical TLC samples were mixed, followed by subsequent pencil column chromatography. The



IUPAC Name: Tetra decane
Molecular formula:CH3(CH2)12CH3
Molecular weight: 198.394 g·mol-1
Physical form: Colourless liquid
Boiling Point: 519.92 [K]
Melting Point: 247.04 [K]

Figure 2. Structure and physical properties of compound 2.

process was carried out until two uncontaminated substances were obtained and their structures confirmed by a range of spectroscopic methods.

2.4. Anti-Leishmanial Activity

The *in vitro* anti-leishmanial experiment was performed using stock solutions at a concentration of 1mg/ml (Shah, Ullah, Ayaz, Sadiq, Hussain, Ali Shah, Shah, Ullah, Ullah, and Ullah 2019). In the 96-well plates, DMSO was used for dilution(Ullah, Shinwari, and Khalil 2017). Promastigotes of L. tropica were grown in medium 199 (Sigma Aldrich) containing penicillin, streptomycin, and 10% fetal bovine serum v/v. 100 microliter of L. tropica log phase culture and 20 microliter of sample were put into wells and incubated at 24 °C for 72 hours. MTT assay was done three days later. The typical medication was glucantime. The graph pad prism was used to calculate the IC50 values.

2.5. Molecular Docking

The phenomenon known as molecular docking was used to explore the interaction that occurs between the isolated chemicals and Trypanothione reductase (TR). The PyRx program was utilized to achieve this purpose (N.U. Khan et al. 2022). The researchers intended to find the specific binding sites shared by the enzyme TR and its ligand over the course of the investigation. A complex was created between the receptor and the active sites of each molecule within the framework of this automated docking procedure, resulting in the development of structural complexes in both two and three dimensions. The best possible binding conformation and associated binding affinities, given in kilocalories per mole, were calculated.

2.6. Statistical Analysis

The experimental findings were reported as the mean value ± the standard deviation, and the experiment was performed three times.

3. Results

3.1. Structure Elucidation of Compound 1 (Dodecane)

It was found that the chemical formula was C12H26. The molecule showed the characteristic peaks of a linear hydrocarbon in its 1H-NMR spectra. Pure hexane was used to create the compound, which was inert when exposed to UV light. The existence of two terminal methyl groups is suggested by the occurrence of a peak at 0.85 ppm that integrates six protons. The peak had a clear J value of 7.5 Hz and appeared as a triplet. The extra signals detected at 1.60-1.25 ppm (18H, m) were associated with long-chain compounds' methylene protons. The molecule's DEPT spectrum showed that it included 12 carbons total—two methyl and ten methylene carbons (Figure S1-S5). The molecule was identified as dodecane by evaluating the spectrum data and

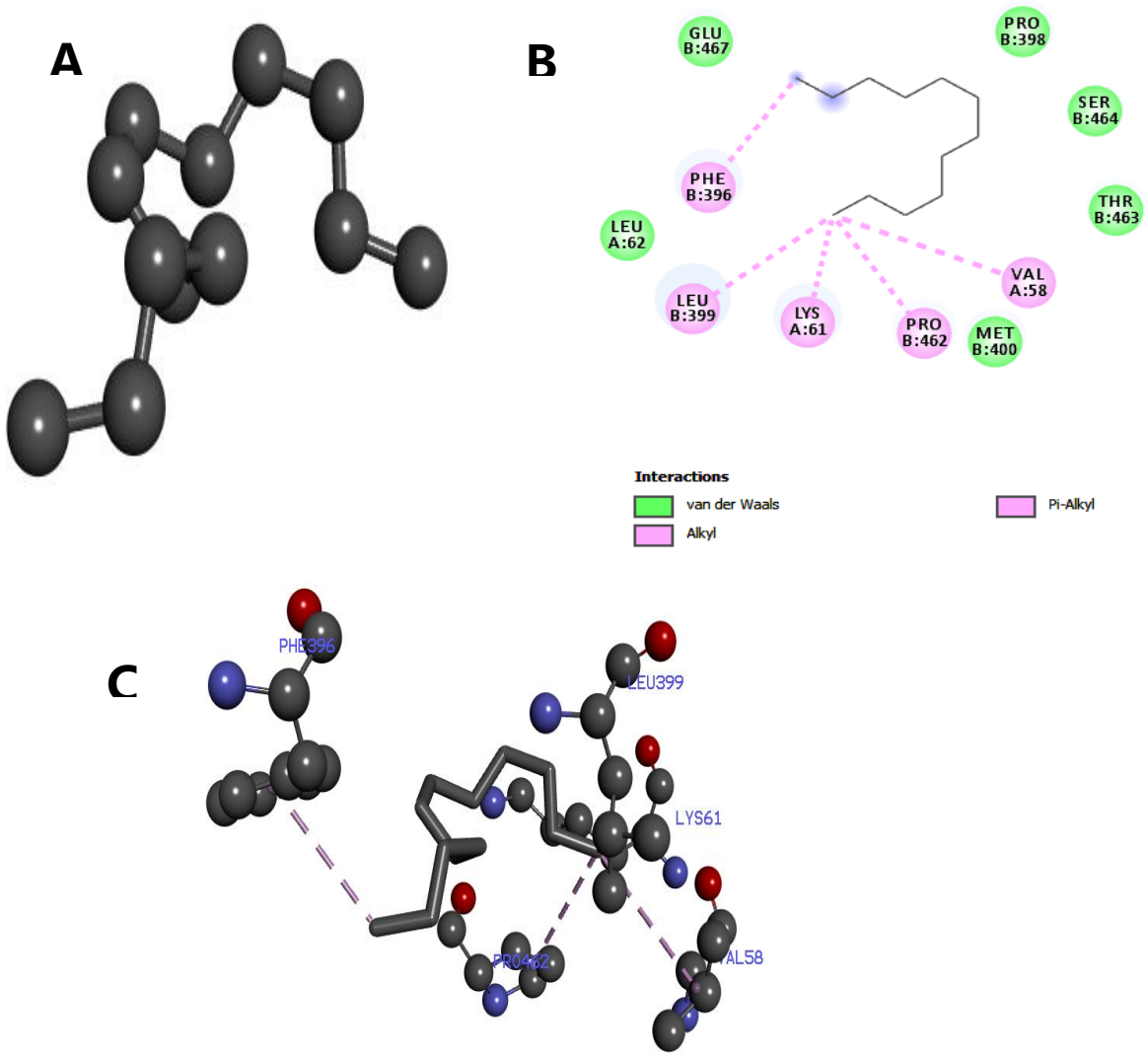


Figure 3: A. Best pose of the compound 1B. 2D molecular docking complex of compound 1 and target C. 3D structural docking complex.

cross-referencing it with previously published research (Figure 1).

3.2. Structure Elucidation of Compound 2 (Tetradecane)

The formula for the compound was found to be C14H30. The molecule showed the characteristic peaks of a linear hydrocarbon in its 1H-NMR spectra. Pure hexane was used to create the compound, which lacked UV activity. The existence of two terminal methyl groups is suggested by the occurrence of a peak at 0.87 ppm that integrates six protons. The peak had a unique J value of 7.5 Hz and appeared as a triplet. The extra upfield signals at 1.5(6H, m) and 1.39-1.20ppm (18H, m) were from protons in long carbon chains that were found in methylene. The molecule's DEPT spectrum showed that it included 14 carbons total—two methyl and twelve methylene carbons(Figure S5-S10). Tetradecane was identified as the drug by evaluating the spectrum data and cross-referencing it with previously published research (Figure 2).

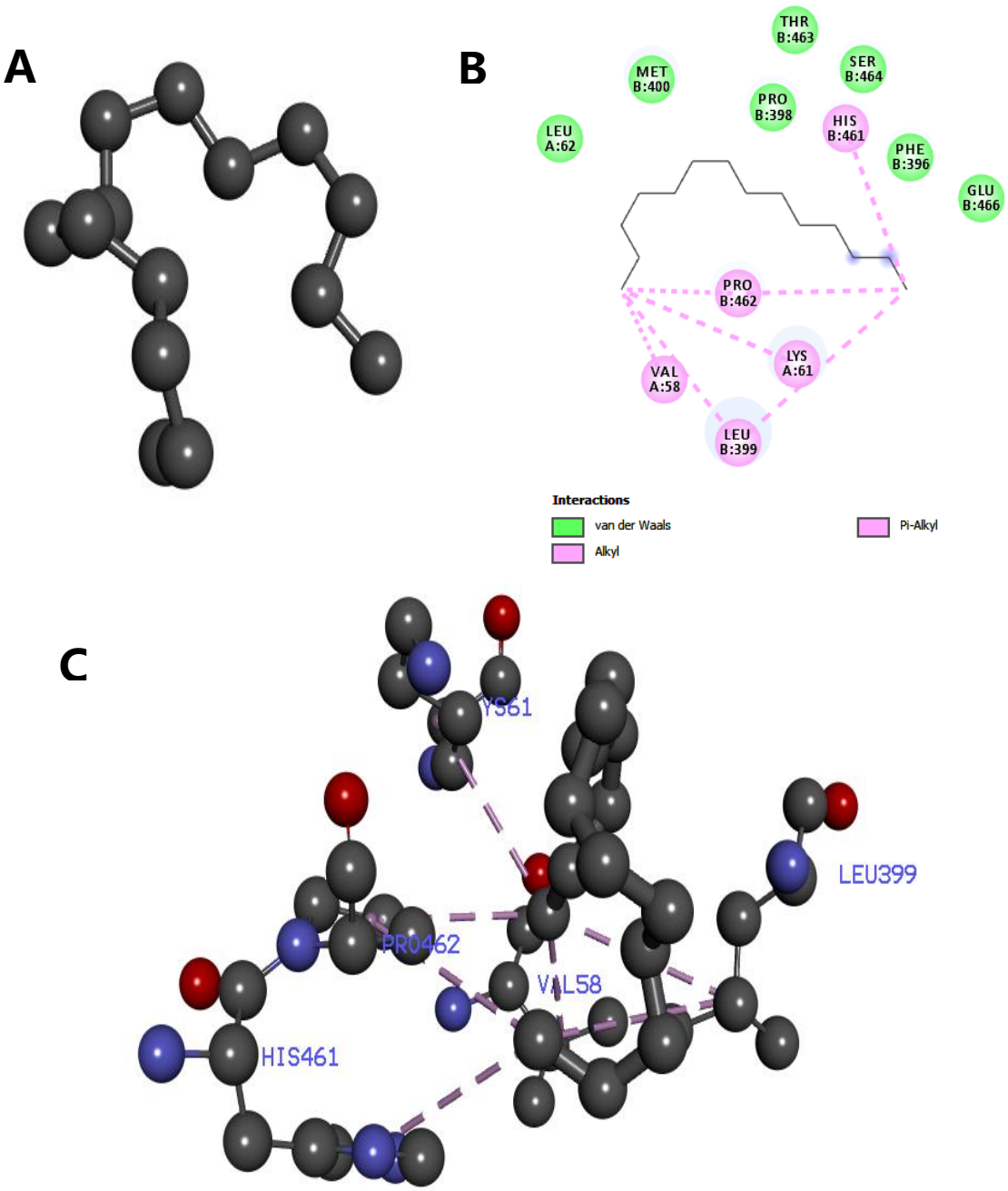


Figure 4: A. Best pose of compound 2, B. 2D molecular docking complex of compound 2 and target, C.3D structural docking complex.

3.3. Antileishmanial Activity of Isolated Compounds

The isolated chemical was subjected to *in vitro* testing to determine its potential for anti-leishmanial action. Column chromatography was used to separate the most bioactive ethyl acetate fraction into three subfractions 5%, 10%, and 15%. A 5% ethyl acetate soluble subfraction was chosen for further processing, yielding three pure chemicals, Dodecane, and Tetra decane which

Table 1. Antileishmanial activity of isolated compounds. Standard drug; Glucantime LD_{50} = 5.33 ± 0.07 µg/mL.

Percent inhibition				IC_{50} of the standard
Compound	**1000µg/ml**	**500µg/ml**	**250µg/mL**	**IC_{50} µg/ml**
Dodecane	56.86	44.89	28.19	0.673
Tetra decane	62.84	41.67	21.77	0.685

Table 2. Bonding energies of Dodecane, 7-aminotridecane, and Tetradecane.

Compound	Binding energy kcal/mol
Dodecane	-4.5 kcal/mol
Tetradecane	-4.7 kcal/mol

were separated at pure n-hexane (Table 1). Although the compounds exhibit moderate activity against *L. tropica*, they may be tested for other strains of leishmania such as *L. donovani* and *L. braziliensis.*

3.4. Molecular Docking of Compound 1

Molecular docking was done on TR with all substances. TR is an enzyme that the parasite needs to stay alive. It works in the same way that glutathione reductase does in animals. The bug dies because of reactive stress when the enzyme gets blocked. Docking was used to find the best position, binding energies, and types of chemical bonds for the molecules. It was possible to get both two-dimensional and three-dimensional shapes. When dodecane formed a complex with TR, it took on a clear two-dimensional shape that was shaped by different types of interactions, such as alkyl bonds and pi alkyl bonds. The amino acid residues of TR and the chemical interacted with each other at the terminal methyl group that was found at both ends. On one end of Dodecane, phenylalanine (PHE B:394) made an alkyl bond with the terminal methyl group. On the other end, leucine (LEU B:399), lysine (LYS B:61), proline (PRO B:462), and valine (VAL B:58) did the same. The lowest binding energy of compound one, -4.5 kcal/mol, showed that it had a strong preference for leishmanial TR. The two-dimensional structures of chemical 1 are shown in Figure 3, which shows that it has alkyl and pi alkyl bonds.

3.5. Molecular Docking of Compound 2

The enzyme ligand complex was studied using TR and Compound 3 (tetra decane). The docking study revealed that there was an alkyl and pi-alkyl type of bonding present between the compound and target site residues. The terminal methyl groups of the compound showed a binding affinity with Valine (VAL B:58), Proline (PRO B:462), Lysine (LYS A:63), Leucine (LEU B:399), and Histidine (HIS B:46). Best docking site was determined and lowest binding energy was calculated which was -4.7 kcal/mol. The 2D and 3D complexes between the target receptor and compound are shown in Figure 4 and binding affinity is given in Table 2.

4. Discussion

Plants have been shown to have secondary metabolites with varied molecular structures that provide anti-leishmanial effects. The prominent plant-derived chemicals that exhibit substantial anti-leishmanial action include indole alkaloids, naphthyl, and bisbenzylisoquinolines, benzoquinolizidines, as well as terpenoids such as diterpenes, triterpenes, and sesquiterpenes. The

compounds included include steroids, saponins, and flavonoids (particularly isoflavones and chalcones) (Passero et al. 2013).

Extracts from the leaves and berries of *Solanum aculeastrum* were explored for the Isolation of volatile Compounds using the Clevenger apparatus and GC-MS analysis. The obtained compounds were mainly straight-chain hydrocarbons from C_{12}-C_{29}. A total of 21 compounds were obtained from the leaves of the plant mainly comprised of aldehydes (nonanal and 9,17-Octadecadienal) and alkanes (predominantly nonane and decane). Some aromatic compounds, Fatty acids and esters, and miscellaneous compounds (ophthalmic acid) were also obtained. A total of 16 compounds were obtained from the n-hexane fraction of the plant methanolic crude extract yielding straight-chain alkanes, predominantly Undecane, Tetradecane, tridecane, and Dodecane (Koduru et al. 2006). Gas chromatography-mass spectrometry was used to determine the volatile oil from *Allium tuberosum* and determined that Chinese chives (*Allium tuberosum* Rottler) release the following volatiles: methyl allyl disulfide, β-myrcene, cis-ocimene, diallyl disulfide, nonane, n-dodecane, n-tetradecane, and n-hexadecane (Yang et al. 2019). The plant *Typhonium flagelliforme* of the Araceae family was investigated and many compounds including methyl esters of hexadecanoic acid, octadecanoic acid, 9-octadecenoic acid, and 9,12-octadecadienoic acid were identified. Straight chain alkanes including dodecane, tridecane, tetradecane, pentadecane, hexadecane, heptadecane, octadecane, nonadecane, and eicosane were also obtained. For alternative cancer therapy, the n-hexane extract of the plant was in vitro investigated against the P388 murine leukemia cells which showed positive results (Choo et al. 2001).

Ifloga spicata is conventionally used for the management of skin conditions such as dermatosis and allergies and in the form of a decoction for heart ailments. The methanolic extract and its subsequent chloroform, ethyl acetate, n-hexane, and aqueous fractions have previously been assessed for their effectiveness against leishmaniasis. The phytochemical screening revealed the presence of all the examined elements, including tannins, flavonoids, saponins, glycosides, terpenoids, alkaloids, and phenolic compounds. The methanolic extract and its derived fractions of *Ifloga spicata* showed anti-leishmanial efficacy. Due to its superior antileishmanial potential, ethyl acetate is an excellent candidate for future investigation (Shah, Ullah, Ayaz, Wahab, et al. 2019).

The ethyl acetate-soluble portion of *Ifloga spicata* was separated by column chromatography. Starting with pure n-hexane, solvent polarity was progressively raised. The isolated compound revealed the chemical formula as C12H26 and C14H30. Both compounds' 1H-NMR spectra revealed the typical peaks of a straight-chain hydrocarbon. The molecule was identified as Dodecane, and Tetradecane, based on this spectrum data and a comparison with the literature.

L. tropica promastigotes in pure cultures were used to evaluate both drugs. Following a 72-hour incubation period, the test compounds had a dose-dependent impact on promastigotes' development, with LD50 values of 0.673 μg/mL for compound 1 and 0.685 μg/mL for compound 2. In the experiment, glucantime served as a positive control (Table 1). Glucantime's LD50 value was found to be 5.33 ± 0.07 μg/mL. There are two different morphological variants of leishmania: promastigote and amastigote. Because of its short culturing period, the promastigote stage is often the subject of anti-leishmanial research (Olivier, Gregory, and Forget 2005). According to the research, both compounds exhibit promising anti-promastigote properties. A previous study found that methyl 3,4-dihydroxybenzoate from *Asphodelus microcarpus* possesses leishmanicidal activity (LD50 = 33.2 μg/mL) against *L. donovani* promastigotes (Ghoneim et al. 2014).

Molecular docking was performed for all compounds (Ali, Khan, et al. 2022) with TR. TR is

an enzyme necessary for parasite survival (Mesías et al. 2019). It is an analog of glutathione reductase in mammals. The inhibition of this enzyme leads to the parasite death as a result of oxidative stress. All the compounds were docked for the best pose, binding energies, and chemical bond types (Ali, Fang, et al. 2022). Two-dimensional and three-dimensional structures were obtained. Dodecane after forming a complex with TR virtually, the two dimensional conformation showed different types of interactions i.e. alkyl bonds and pi alkyl bonds. The bonds between different amino acid residues of TR and the compound were observed at the terminal methyl group at both ends. Phenylalanine (PHE B:394) formed an alkyl bond with the terminal methyl group at one end of Dodecane while leucine (LEU B:399), lysine (LYS B:61), Proline (PRO B:462) and valine (VAL B:58) formed at the other end. The lowest binding energy -4.5 kcal/mol was recorded in compound one showing its strong affinity with leishmanial TR. The two-dimensional structures presenting alkyl and pi alkyl bonds of compound 1 with TR are given in Figure 3.

TR and Compound 2 (tetra decane) were used to investigate the enzyme ligand complex. The docking analysis found that there was alkyl and pi-alkyl bonding between the chemical and the target site residues. The compound's terminal methyl groups attach to Valine (VAL B:58), Proline (PRO B:462), Lysine (LYS A:63), Leucine (LEU B:399), and Histidine (HIS B:46). The best docking site was identified, and the lowest binding energy was predicted to be -4.7 kcal/mol. Figure 4 depicts the 2D and 3D complexes formed by the target receptor and chemical, and Table 2 shows the binding affinity.

5. Conclusion

Ifloga spicata was chosen as a significant medicinal plant for this investigation. Methanol and its derived fractions were prepared. The ethyl acetate fraction followed purification according to prior literature research. The ethyl acetate soluble fraction from *Ifloga spicata* was subjected to preliminary phytochemical research, resulting in the identification of Dodecane and Tetradecane.

The current work demonstrates that the extracted compounds exhibited antileishmanial action against *L. tropica* promastigotes, which was then validated using molecular docking. The cell death in *Leishmania promastigotes* occurred because the parasite enzyme TR was inhibited. This inhibition was validated by studying the binding affinities between the chemical and the amino acid residues of the enzyme TR during docking tests.

Conflict of Interest

The authors declare that they have no competing interests.

Funding

NA.

Study Approval

The study was approved by the Department of Pharmacy, Kohat University of Science & Technology, Kohat, Pakistan.

Consent Forms

NA.

Authors Contribution

MS, FA, SH, ZBM, and FU performed experimental work, data collection and evaluation, literature search, and manuscript preparation. SAK and SMS supervised the research work and refined the manuscript for publication. The authors read and approved the final manuscript for publication.

Data Availability

All the relevant data of this manuscript is available with the authors.

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