World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 7.409

SYNTHESIS, CHARACTERIZATION AND IN SILICO EVALUATION OF NOVEL AZO DERIVATIVE FROM THYMOL AND SULPHANILIC ACID

[1]*Balachandar S., [2]Alagarraja M., [2]Indumathi M., [2]Ranjith M., [2]Kadal Selvi S., [2]Gunaseelan S., [2]Mukilan S. and [2]Sasikala R.

[1]Department of Pharmacognosy, United College of Pharmacy, G.Koundampalayam, Periyanaickanpalayam, Coimbatore, Tamilnadu, Coimbatore-641020.
[2]Department of Pharmaceutical Chemistry, United College of Pharmacy, G.Koundampalayam, Periyanaickanpalayam, Coimbatore, Tamilnadu, Coimbatore-641020.

***Corresponding Author: Balachandar S.**
Department of Pharmacognosy, United College of Pharmacy, G.Koundampalayam, Periyanaickanpalayam, Coimbatore, Tamilnadu, Coimbatore-641020.

Article Received on 15/04/2025 **Article Revised on 05/05/2025** **Article Accepted on 26/05/2025**

ABSTRACT
Thymol is known for its strong anti microbial properties, effective against various bacteria and fungi. As thymol has low solubility in water and photo decomposition, the current study is to overcome the disadvantages Thymol is known for its strong antimicrobial properties, effective against various bacteria and fungi. As thymol has low solubility in water and photo-decomposition, the current study is to overcome the disadvantages and level up the potency against microbes. Thymol and sulphanilic acid were coupled with an azo linkage by diazotization and coupling reactions. An orange-red dye (BBH-1) was synthesized in optimum condition. Structure of the compound was confirmed by FTIR and ^{13}C-NMR. Physical properties like melting point and solubility have been established. Docking studies have been performed against protozoal protein 5QQ5 and fungal protein 5FSA in comparison with Metronidazole and Itraconazole as standards, respectively. In which the compound BBH-1 possessed potent anti-protozoal effect over the standard drug. Meanwhile, acceptable antifungal effect have been observed when compared to Itraconazole. Insilico toxicity studies revealed that the compound is extremely safe with LD_{50} around 5000 mg/kg. As per the research outcome, this azo derivative is safe to perform animal studies, and it is suitable for topical application. Further development is encouraged by chemical optimization.

KEYWORDS: Thymol, azo derivative, anti microbial, docking studies.

INTRODUCTION
Azo compounds constitute an important class of organic molecules distinguished by the presence of the azo (-N=N-) functional group.[1] These compounds exhibit remarkable versatility, with applications spanning dyes, pigments, pharmaceuticals, and advanced material sciences. Due to their diverse biological activities, particularly in antimicrobial and anti parasitic treatments, research on azo compounds has gained significant traction.[2] Their structural flexibility allows for modifications that enhance pharmacological properties, making them valuable candidates for drug development.[3] Medicinal chemistry focuses on designing and developing compounds with therapeutic potential, and azo compounds play a pivotal role in this field due to their bioactive nature.[4,5] Numerous azo derivatives have demonstrated effectiveness as antimicrobial, anticancer, and anti-inflammatory agents.[6,7,8] Their mode of action frequently involves interacttions with biological targets such as enzymes and receptors via the azo linkage. Additionally, azo prodrugs serve as targeted drug delivery systems, wherein enzymatic reduction in specific physiological conditions releases the active therapeutic agent. The growing issue of antimicrobial resistance underscores the need for novel therapeutic agents.[9,10,11,12,13] Azo compounds have exhibited promising antimicrobial properties against a range of bacterial, fungal, and protozoal pathogens.[14,15,16] Their structural characteristics facilitate selective interactions with microbial enzymes, disrupting essential cellular processes.[6,17] Some azo derivatives, such as sulfonamides, have already been successfully used in clinical treatments for bacterial infections.[10] Moreover, computational drug design and in silico studies have significantly contributed to optimizing azo-based molecules for enhanced therapeutic efficacy. Advancements in computational methods, particularly Computer-Aided Drug Design (CADD), have transformed the development of azo-based pharmaceuticals. Molecular docking and molecular

dynamics simulations help identify potential drug candidates by predicting their interactions with biological targets. Additionally, Quantitative Structure-Activity Relationship (QSAR) models offer valuable insights into optimizing azo derivatives for improved efficacy and reduced toxicity. The integration of artificial intelligence and machine learning in medicinal chemistry has further accelerated the discovery of novel azo-based therapeutics.[4,5,18] This study focuses on the synthesis, characterization, and biological evaluation of novel azo compounds with potential antimicrobial and therapeutic applications. It employs computational approaches to assess the safety and effectiveness of these compounds.[9,12,19,20] By utilizing modern drug design techniques, this research aims to contribute to the development of effective antimicrobial agents and expand the role of azo chemistry in medicinal applications. In summary, azo compounds offer significant potential in pharmaceutical sciences, serving as a foundation for innovative drug development. Their chemical adaptability, combined with advances in computational methodologies, creates new opportunities for discovering therapeutic agents. This research seeks to provide valuable insights into the potential of azo compounds in addressing current medical challenges.

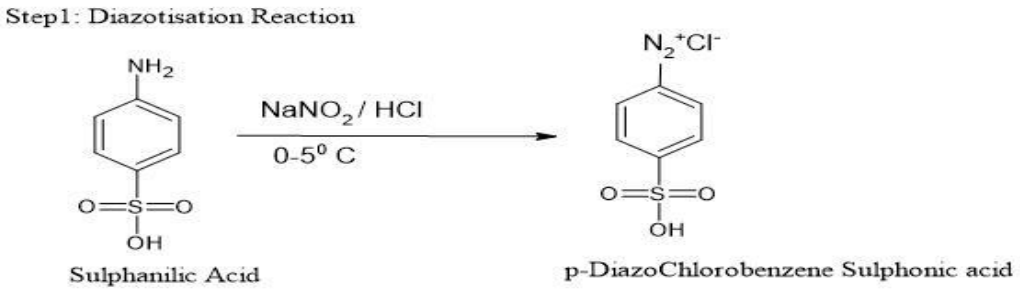
MATERIALS AND METHODS

Materials

This research utilized various chemicals and reagents, including sulphanilic acid, sodium nitrite, hydrochloric acid, thymol, and sodium hydroxide, for the synthesis of azo compounds. Multiple solvents, such as water, ethanol, ethyl acetate, benzene, chloroform, methanol, and n-hexane, were used for solubility and purification studies. The crude product was purified using solvent fractionation, recrystallization with ethanol, and chromatographic techniques such as column chromatography and thin-layer chromatography (TLC) and melting point using Cole Parmer MP-250 to ensure purity. The characterization of the synthesized azo compounds was performed using various spectroscopic techniques. UV-Visible spectrophotometry was employed to examine electronic transitions within the 200–800 nm range. Infrared (FTIR) spectroscopy was used to identify functional groups by scanning in the 4000–400 cm^{-1} range using Jasco FTIR 4X. Additionally, Nuclear Magnetic Resonance (NMR) spectroscopy, including both ^{1}H and ^{13}C NMR, was conducted using a Bruker Ascend™ 500 spectrometer with DMSO-d6 as the solvent. Computational studies were carried out to assess the biological potential of the synthesized compounds. Molecular docking was performed using PyRx software to predict ligand-receptor interactions, while BIOVIA Discovery Studio was utilized for visualizing binding affinities. Furthermore, pharmacokinetic properties, including absorption, distribution, metabolism, and excretion (ADME), were predicted using SWISSADME software. Toxicity assessments were conducted using ProTox-3.0 to evaluate potential risks. By integrating both experimental and computational approaches, this research provided a comprehensive understanding of the structural and biological characteristics of the newly synthesized azo derivatives.

Methods

The synthesis of azo compounds involved a sequence of chemical reactions, purification procedures, and characterization techniques. Initially, thymol of 2g was melted and dissolved in a 10% sodium hydroxide solution 30ml while being maintained in an ice bath. At the same time, sulphanilic acid 1.73ml was dissolved in concentrated hydrochloric acid 4ml and reacted with sodium nitrite1g to generate the diazonium salt. This solution was then gradually added dropwise to the thymol mixture, ensuring that the temperature remained between 0–5°C. The reaction mixture was continuously stirred, with 10% sodium hydroxide added incrementally to promote the coupling reaction.



Step: 2 Coupling reaction

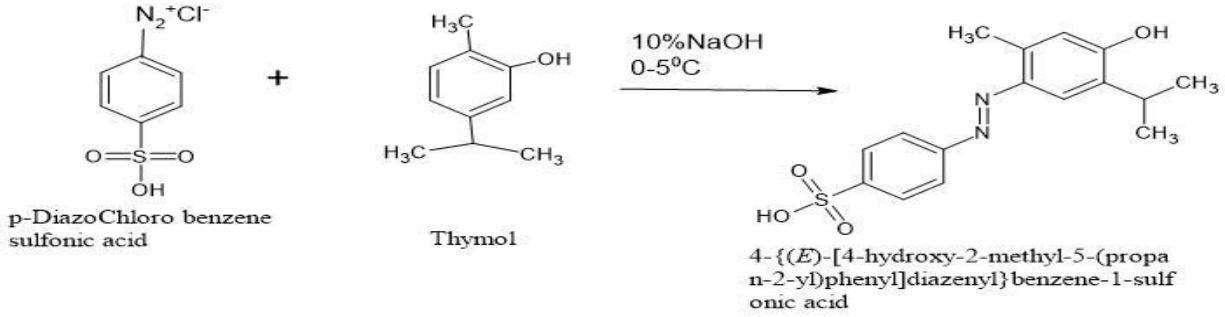


Figure 1: Synthesis Scheme.

The resulting azo compound was precipitated, neutralized with water, filtered, and dried. Purification of the crude product was carried out through solvent fractionation using n-hexane, followed by recrystallization with ethanol to eliminate impurities. Further refinement was achieved via column chromatography, utilizing silica gel (100–200 mesh) as the stationary phase. A gradient solvent system comprising n-hexane and ethanol (9:1), followed by ethyl acetate and ethanol (1:1), was employed for elution. The purified fractions were collected, evaporated to dryness, and analyzed using thin-layer chromatography (TLC) on a silica gel plate with ethanol:benzene (3:7) as the mobile phase. Retention factor (Rf) values were measured to verify purity. Characterization of the synthesized azo compounds was conducted using various spectroscopic techniques. UV-Visible spectrophotometry was utilized to examine electronic transitions within the 200–800 nm range. Fourier Transform Infrared (FTIR) spectroscopy was applied to identify functional groups within the 4000–400 cm^{-1} range. Additionally, Nuclear Magnetic Resonance (NMR) spectroscopy, including ^{13}C NMR, was performed using a Bruker Ascend™ 500 spectrometer with DMSO-d6 as the solvent. Computational studies were incorporated to assess the biological potential of the synthesized azo compounds. Molecular docking analysis was carried out using PyRx software to evaluate ligand-receptor interactions, while BIOVIA Discovery Studio was employed for visualization and affinity analysis. Pharmacokinetic properties, such as absorption, distribution, metabolism, and excretion (ADME), were predicted using SWISSADME software. Additionally, toxicity evaluations were conducted using ProTox-3.0 to assess potential safety risks. By integrating both experimental and computational approaches, this study provided a comprehensive evaluation of the structural and biological properties of the synthesized azo derivatives.

RESULTS AND DISCUSSION

Preliminary Studies

The synthesized azo compound, was obtained as a crystalline solid. The percentage yield of the compound was found to be 55.68% w/w, which is relatively high compared to the theoretical yield, confirming a successful synthesis. Table 1 summarizes the general characteristics of the synthesized compound, including molecular formula, molecular weight, and state.

Table 1: General Characteristics of Synthesized Compound.

S.No	Molecular Formula	Molecular Weight	State
1	C16H18N2O4S	334.39 g/mol	Crystalline

The compound exhibited orange red colouration, confirming the presence of an extended conjugated system.

Figure 2: Synthesized sample.

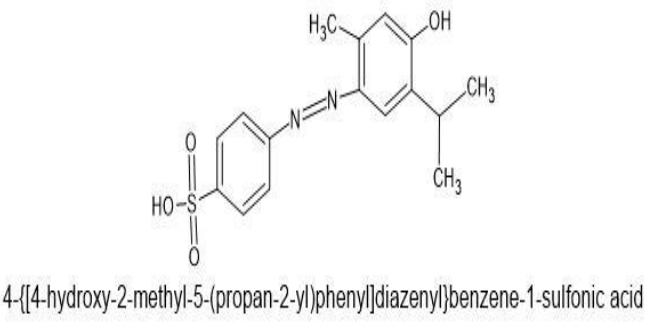


Figure 3: 2D structure of compound.

Solubility Profile

The solubility studies indicate that compound is soluble in water, ethanol, and methanol, while it is insoluble in benzene and n-hexane, suggesting potential hydrophilic interactions.

Table 2: Solubility Profile.

S.No	Solvent	Solubility
1	Water	Soluble
2	Ethanol	Soluble
3	Benzene	Insoluble
4	n-Hexane	Insoluble

Structural Analysis

The UV-visible spectrophotometric analysis of BBH-1 was performed in ethanol solvent within the range 200–800 nm. The absorption maximum (λ max) was observed at 371.5 nm, indicating a strong π-π* transition due to the presence of an extended conjugated system.

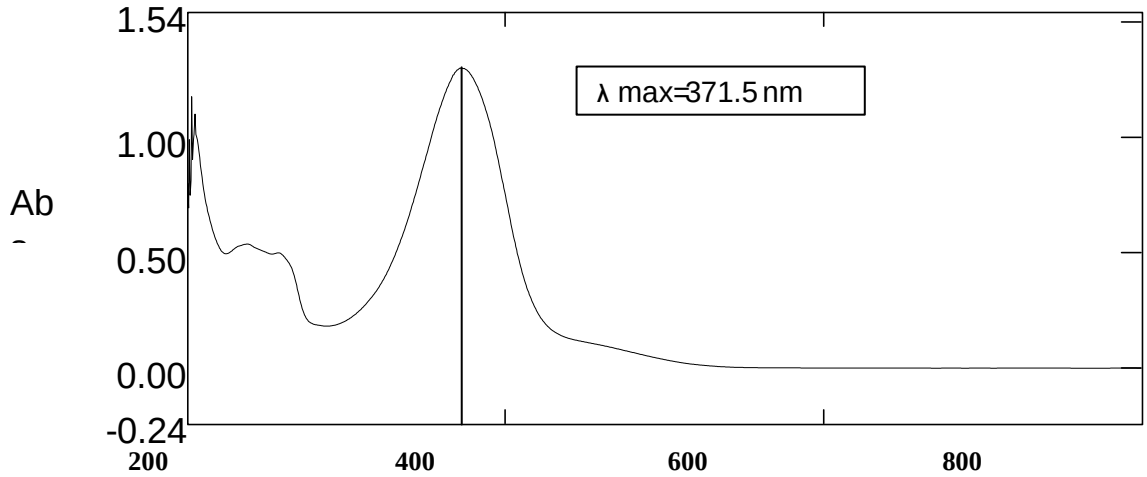


Figure 4: UV visible Absorption Spectrum of compound.

The IR spectral analysis of sample reveals key functional groups within its molecular structure. A strong absorption at 3439.42 cm^{-1} corresponds to -OH stretching, indicating the presence of hydroxyl groups, likely involved in hydrogen bonding. The peaks at 2966.95 cm^{-1} suggest aliphatic C-H stretching, pointing to the existence of alkyl chains. The absorption at 1634.38 cm^{-1} is characteristic of C=C stretching in aromatic systems, confirming an aromatic ring. The presence of an azo (-N=N-) group is supported by a strong band at 1512.88 cm^{-1}, while the C-N stretch at 1319.07 cm^{-1} further indicates nitrogen-containing functionalities. Additionally, two distinct S=O stretching vibrations appear: asymmetric stretching at 1387.53 cm^{-1} and symmetric stretching at 1195.65 cm^{-1}, which are characteristic of sulfone ($-SO_2$) groups. The C-S (aromatic) stretching at 1040.41 cm^{-1} and the C-O stretching at 1127.19 cm^{-1} further confirm the presence of sulfur and oxygen functionalities.

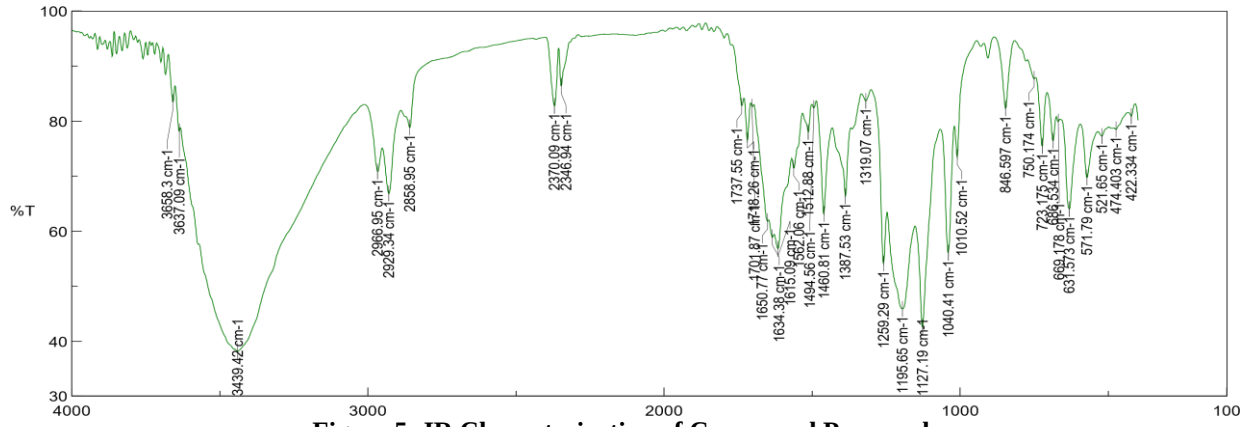


Figure 5: IR Characterization of Compound Prepared.

The provided information is a Carbon-13 Nuclear Magnetic Resonance (^{13}C NMR) spectrum that displays the number of protons bonded to each carbon in a molecule as well as chemical shifts (ppm). The ppm values show the electrical environment of the carbon atoms in the structure, while the Atom Number column

probably relates to particular carbon atoms. According to the data, the carbons at 17 and 18 ppm are linked to six protons and exhibit a chemical shift of 1.18–1.19 ppm, indicating that they are methyl groups (-CH_3) in a somewhat nonpolar environment, most likely connected to sp^3 hybridised carbon. Similarly, a -CH_2- group is indicated by the carbon being linked to two protons at 15 and 23 ppm (with a shift of 2.51 ppm). Three protons make up the carbon at 19 ppm with a shift of 2.59 ppm; this is probably a methyl group in a slightly different electrical environment. Because they contain fewer protons, the carbons at 16 ppm (3.30 ppm) and 10, 13, 2, 3, 5, and 6 ppm (with shifts of 6.81, 7.58, and 7.76 ppm) are more likely to be attached to electronegative atoms or be in aromatic/conjugated systems. The consistency of the structure is confirmed by the sum of 18 protons bonded to these carbons. The influence of shielding effects, hybridisation, and electronegativity inside the molecule is reflected in the changes in chemical shifts.

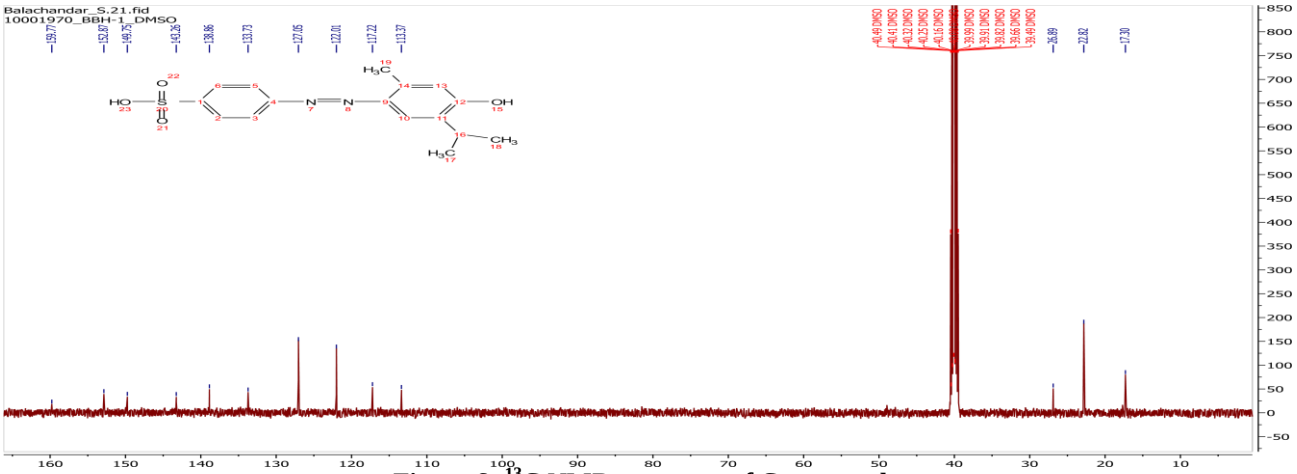


Figure 6: ^{13}C NMR spectrum of Compound.

Thin Layer Chromatography (TLC) Analysis

The TLC analysis was performed using ethanol as a solvent system. A single spot with an Rf value of 0.52 was observed under UV light, confirming the purity of the synthesized compound.

Table 3: Thin Layer Chromatography and Rf Value.

S.No	Colour of Spot	Rf Value
1	Yellow	0.52

Melting Point Determination

The melting point of compound was recorded as 227–229°C, indicating thermal stability. A small amount of sublimation was observed, as seen in the melting point analysis.

Molecular Docking Studies

The docking studies were conducted to analyze the interaction of BBH-1 with antifungal and antiprotozoal targets. Docking studies with Trypanosoma cruzi (PDB ID: 5QQ5) revealed a binding affinity of -8.6 kcal/mol, suggesting strong interactions. Interaction studies with Candida albicans (PDB ID: 5FSA) showed a binding affinity of -7.6 kcal/mol, indicating potential antifungal activity. The binding affinity of BBH-1 was compared with standard antifungal and antiprotozoal drugs. Metronidazole, a known antiprotozoal agent, had a binding affinity of -4.9 kcal/mol, whereas Itraconazole, a standard antifungal drug, had an affinity of -10.2 kcal/mol.

Table 6: Comparison of Binding Affinity with Standard Drugs.

S.No	Protein ID	Ligand ID	Binding Affinity (kcal/mol)	Standard Drug	Binding Affinity (kcal/mol)
1	5QQ5	BBH-1	-8.6	Metronidazole	-4.9
2	5FSA	BBH-1	-7.6	Itraconazole	-10.2

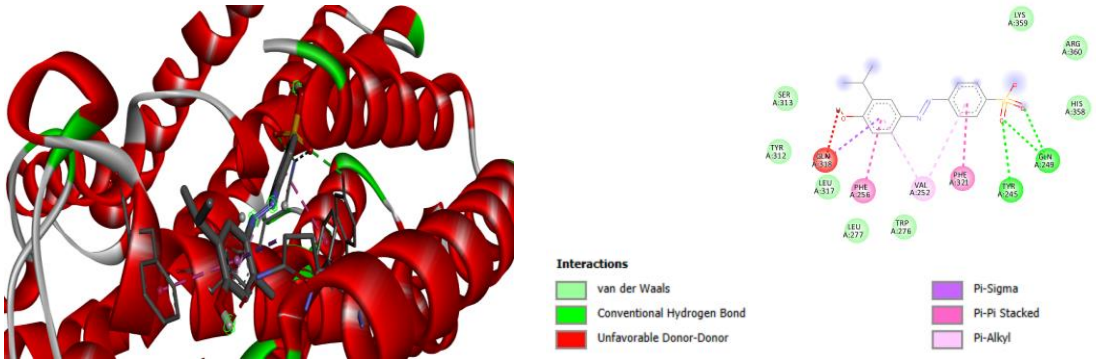


Figure 7: Interactions of ligand with Trypanosoma cruzi (PDB ID: 5QQ5) in 3D and 2D.

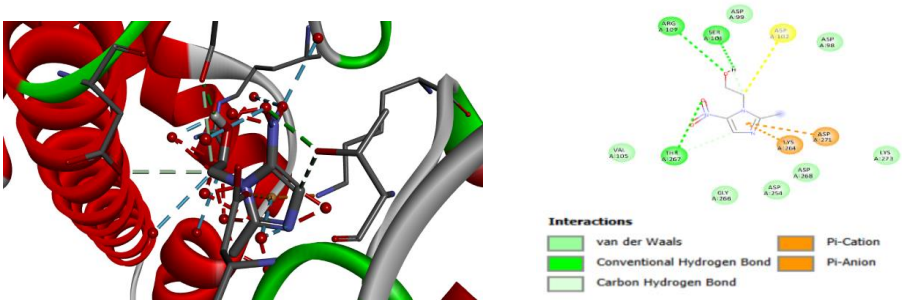


Figure 8: Interactions of metronidazole with Trypanosoma cruzi (PDB ID: 5QQ5) in 3D and 2D.

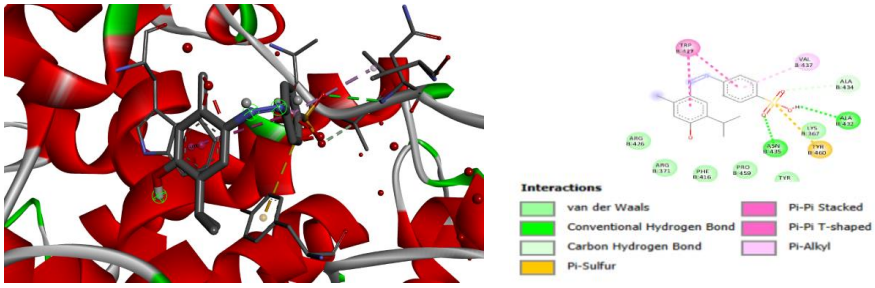


Figure 9: Interactions of Ligand with Candida albicans (PDB ID: 5FSA) in 3D and 2D.

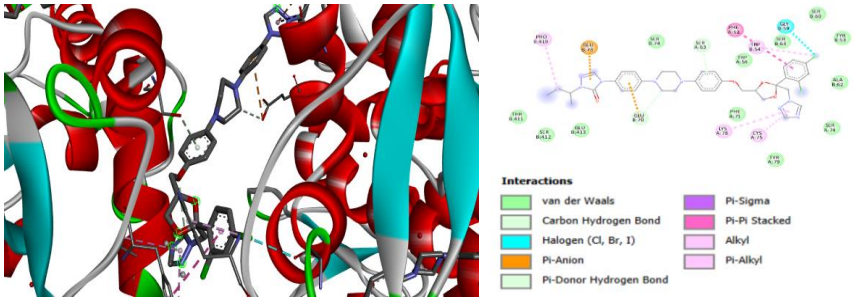


Figure 10: Interactions of itraconazole with Candida albicans (PDB ID: 5FSA) in 3D and 2D.

Toxicity Studies

Toxicity analysis using ProTox-3.0 predicted for sample to be non-toxic with an LD50 value of 5000mg/kg**.**

Table 7: Toxicity Studies.

Toxicity Type	Predicted Result	Probability	LD50 (mg/kg)	Toxicity Class
Hepatotoxicity	Inactive	0.65	5000	5
Nephrotoxicity	Inactive	0.55		
Mutagenicity	Inactive	0.70		
Cytotoxicity	Inactive	0.78		

CONCLUSION

The synthesized compound demonstrated good solubility, thermal stability, and structural integrity, confirmed by UV, IR, and NMR analyses. Molecular docking studies revealed strong binding affinity against Trypanosoma cruzi (-8.6 kcal/mol) and Candida albicans (-7.6 kcal/mol), indicating potential antimicrobial activity. Compared to standard drugs, sample showed better affinity than metronidazole but slightly lower than itraconazole, suggesting its potential as an antifungal and antiprotozoal agent. Additionally, toxicity predictions classified the compound as non-toxic (LD50: 5000 mg/kg), supporting its safety profile. These findings highlight sample as a promising lead compound for novel antimicrobial drug development, warranting further in vitro and in vivo studies to confirm its therapeutic potential.

ACKNOWLEDGEMENT

We would like to extend our sincere thanks to United College of Pharmacy, G.koundampalayam, Periyanaickanpalayam, Coimbatore, Tamilnadu for assistance in carrying out the research. We would like to extend our sincere gratitude to the Department of Pharmaceutical Engineering and Technology and Central Instrument Facility of Indian Institute of Technology (BHU), Varanasi, UP for the assistance in the spectral studies.

REFERENCES

1. Ali, Y., Hamid, S.A., Rashid, U., 2018. Biomedical Applications of Aromatic Azo Compounds. Mini Rev. Med. Chem, 18: 1548–1558. https://doi.org/10.2174/1389557518666180524113111
2. Azo Polymers: Synthesis, Functions and Applications | SpringerLink [WWW Document], n.d. URL https://link.springer.com/book/10.1007/978-3-662-53424-3 (accessed 2.12.25).
3. Benkhaya, S., M'rabet, S., El Harfi, A., 2020. Classifications, properties, recent synthesis and applications of azo dyes. Heliyon, 6: e03271. https://doi.org/10.1016/j.heliyon.2020.e03271
4. Çanakçı, D., 2020. Synthesis, Spectroscopic, Thermodynamics and Kinetics Analysis Study of Novel Polymers Containing Various Azo Chromophore. Sci. Rep, 10: 477. https://doi.org/10.1038/s41598-019-57264-3
5. Chung, K.-T., 2016. Azo dyes and human health: A review. J. Environ. Sci. Health Part C., 34: 233–261. https://doi.org/10.1080/10590501.2016.1236602
6. Dembitsky, V.M., Gloriozova, T.A., Poroikov, V.V., 2017. Pharmacological and Predicted Activities of Natural Azo Compounds. Nat. Prod. Bioprospecting, 7: 151–169. https://doi.org/10.1007/s13659-016-0117-3
7. Dragan, D., Goran, A., Aleksandra, M., Dragan, M., Miodrag, S., 2016. ANTI-MICROBIAL AND ANTI-AMOEBIC ACTIVITY SOME AZOMETHINES - POTENTIAL TEXTILE DYESTUFFS. | EBSCOhost [WWW Document]. URL https://openurl.ebsco.com/contentitem/gcd:118477399?sid=ebsco:plink:crawler&id=ebsco:gcd:118477399 (accessed 2.12.25).
8. Esmatabadi, M.J.D., Bozorgmehr, A., Hajjari, S.N., Sombolestani, A.S., Malekshahi, Z.V., Sadeghizadeh, M., 2017. Review of new insights into antimicrobial agents. Cell. Mol. Biol, 63: 40–48. https://doi.org/10.14715/cmb/2017.63.2.6
9. Jyoti R. Gadhe*, R.A., 2024. GLIMPSES ON VALIDATION OF ASSAY METHOD FOR ESTIMATION OF ITRACONAZOLE IN ITRACONAZOLE TABLET DOSAGE FORM BY UV-SPECTROSCOPY. https://doi.org/10.5281/ZENODO.11561616
10. Moellering, R.C., 2011. Discovering new antimicrobial agents. Int. J. Antimicrob. Agents, 37: 2–9. https://doi.org/10.1016/j.ijantimicag.2010.08.018
11. Noser, A.A., Alkhikany, A.-A.-B., Salem, M.M., Salam, H.A.A.E., 2025. Discovery of novel azo pyrimidinone derivatives as B-cell lymphoma-2 inhibitors with potential antineoplastic activity: Synthesis, characterization, in-silico, and in-vitro studies. J. Mol. Struct, 1323: 140783. https://doi.org/10.1016/j.molstruc.2024.140783
12. Options Méditerranéennes en ligne - Collection numérique - Antimicrobial agents in aquaculture: practice, needs and issues [WWW Document], n.d. URL https://om.ciheam.org/article.php?IDPDF=801061 (accessed 2.12.25).
13. (PDF) DIAZOTISED SULPHANILIC ACID REAGENT FOR THE DETERMINATION OF THIAMINE IN AQUEOUS SOLUTION-APPLICATION TO PHARMACEUTICAL PREPARATIONS [WWW Document], n.d. URL https://www.researchgate.net/publication/328789879_DIAZOTISED_SULPHANILIC_ACID_REAGENT_FOR_THE_DETERMINATION_OF_THIAMINE_IN_AQUEOUS_SOLUTION-APPLICATION_TO_PHARMACEUTICAL_PREPARATIONS?enrichId=rgreq-6f0337984dd689b3278070d35f542a9d-XXX&enrichSource=Y292ZXJQYWdlOzMyODc4OTg3OTtBUzo2OTAzNjU2Njk1ODg5OTVAMTU0MTYwNzQxMDkwNg%3D%3D&el=1_x_2&_esc=publicationCoverPdf (accessed 2.12.25).
14. Principles of early drug discovery - Hughes - 2011 - British Journal of Pharmacology - Wiley Online Library [WWW Document], n.d. URL https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/j.1476-5381.2010.01127.x (accessed 2.12.25).
15. Roldo, M., Barbu, E., Brown, J.F., Laight, D.W., Smart, J.D., Tsibouklis, J., 2007. Azo compounds in colon-specific drug delivery. Expert Opin. Drug Deliv, 4: 547–560. https://doi.org/10.1517/17425247.4.5.547

16. Shchegol'kov, E.V., Khudina, O.G., Anikina, L.V., Burgart, Ya.V., Saloutin, V.I., 2006. Synthesis, analgesic and antipyretic activity of 2-(antipyrin-4-yl)hydrazones of 1,2,3-triketones and their derivatives. Pharm. Chem. J., 40: 373–376. https://doi.org/10.1007/s11094-006-0130-7
17. Sıdır, Y.G., Sıdır, İ., Berber, H., Taşal, E., 2011. UV-spectral changes for some azo compounds in the presence of different solvents. J. Mol. Liq, 162: 148–154. https://doi.org/10.1016/j.molliq.2011.07.002
18. STUDY OF AZO COMPOUNDS DERIVED FROM P-CRESOL LIGAND: SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL ACTIVITY | Journal of Experimental Biology and Agricultural Sciences [WWW Document], n.d. URL https://jebas.org/ojs/index.php/jebas/article/view/42 (accessed 2.12.25).
19. The Handbook of Medicinal Chemistry: Principles and Practice, 2014. The Royal Society of Chemistry.
20. The Medicinal Chemist's Toolbox: An Analysis of Reactions Used in the Pursuit of Drug Candidates | Journal of Medicinal Chemistry [WWW Document], n.d. URL https://pubs.acs.org/doi/10.1021/jm200187y (accessed 2.12.25).