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RESEARCH ARTICLE

Anti-Tuberculosis and Molecular Docking Study of – Rhizomes of *Curcuma caesia*

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Abstract

Objective: To isolate and evaluate bioactive compounds from *Curcuma caesia* rhizomes for its novel potential anti-tuberculosis agents. **Methods**: *Curcuma caesia* rhizomes were meticulously collected and authenticated. Phytochemical screening and GC-MS analyses were conducted to identify the chemical constituents of the plant. Isolation of a bioactive phytoconstituent was achieved, followed by the characterization using HPLC, TLC, and FTIR techniques. The isolated compound was then subjected to biological evaluation, including anti-tuberculosis activity assessment and MTT assay, revealing its potential as a novel anti-tuberculosis agent. **Findings:** Alkaloids, flavonoids, phytosterols, saponins, and phenolics were identified among the chemical components in the early phytochemical screening. Bioactive compounds were extracted using methanol, and the compound, (+)-3-Bromocamphor-8-Sulfonic Acid Ammonium Salts, showed positive for anti-tuberculosis agent. *In silico* docking studies of isolated compounds and proteins showed conventional hydrogen bonds with 8hcr 5sxf. The compound adheres to Lipinski's rule of five, suggesting its potential as a drug-like molecule. **Novelty:** The isolation of novel (+)-3-Bromocamphor-8-sulfonic acid ammonium salts showed a potential anti-tuberculosis role that can act against drug-resistant strains of *Mycobacterium tuberculosis*. Adherence to Lipinski's rule of five suggests that it is a drug-like molecule, emphasizing its potential for further drug development.

Keywords: Curcuma caesia; (+)3Bromocamphor8Sulfonic Acid Ammonium Salts; Antitubercular activity; Mycobacterium tuberculosis; MTT assay

1 Introduction

The bacterium *Mycobacterium tuberculosis* (MTB) is the primary cause of the highly infectious illness tuberculosis (TB). It is still a severe hazard to global health and is

responsible for a large number of fatalities as it stands top ten causes of death globally. It is alarming that approximately 25% of the world's population is infected with MTB. Consequently, TB has surpassed HIV/AIDS as the primary infectious disease-associated cause of death [1]. Extrapulmonary TB can impact other parts of the body even though it primarily affects the lungs (pulmonary TB). The World Health Organization's 2022 report estimates that 2 billion people, or 25% of the world's population, are living with latent tuberculosis infections (LTBI). An estimated 5% to 10% lifetime risk of TB reactivation happens among individuals with LTBI [2]. The incidence of TB increased in the latter half of the 20th century, predominantly in those with HIV infections. [3,4] The primary challenge in the management of TB is the emergence of drug resistance due to irregular or incomplete treatment, particularly when the infection is classified as multidrug-resistant (MDR) TB [5,6]. Because of the widespread problem posed by TB, the pursuit of new alternative active metabolites derived from natural sources becomes a paramount concern.

Curcuma caesia (*C. caesia*), commonly known as moniker black turmeric, stands out as an interesting representative of the Zingiberaceae family. Its rhizomes, valued for their therapeutic benefits, offer remedies for concerns such as sprains and bruises, and also find application in the realm of cosmetics. Additionally, they serve as a carminative, alleviating headaches, and rheumatic pains [7–9]. The adverse impacts triggered by anti-tuberculosis drugs, culminating in the production of detrimental substances like toxic metabolites, relative oxygen species (ROS), and free radicals, raised concerns regarding the impact on the liver [10,11] and have sparked renewed interest in innovating strategies for the development of more potent anti-tuberculosis treatments. Curcumin has surfaced as a promising candidate, offering hepatoprotective potential [12,13]. The primary bioactive elements present in the plants' rhizomes comprise curcumin along with two associated compounds: demethoxycurcumin and bisdemethoxycurcumin [14]. Hence, this study was conducted to isolate and characterize the specific phytoconstituents within the rhizomes of *C. caesia*, unravelling their potential as agents to combat tuberculosis. Furthermore, the docking study was also conducted to explore the possibility of metabolite as a future drug against *Mycobacterium tuberculosis*.

2 Materials & Methodology

2.1 Procurement of *C. caesia* Rhizomes

The fresh rhizomes of *C. caesia* were collected from Ranipool, East Sikkim, India. The rhizomes were identified and authenticated by Dr. P.E. Rajasekharan, Principal Scientist, ICAR- Indian Institute of Horticulture Research, Hesaraghatta Late Post, Bengaluru, as *C. caesia* belonging to the family Zingiberaceae.

2.2 Preparation of Extract

The rhizomes were subjected to a wash to get rid of dust and impurities. This was followed by cutting them finely into small pieces. After they had dried completely, they were coarsely powdered using a mechanical grinder. The resulting dried powder was then used in the subsequent solvent extraction. The extract obtained from methanol was concentrated under reduced pressure, at 40 °C.

2.3 Phytochemical tests

Using established standard methods, phytochemical analysis of the extract to identify compounds such as alkaloids, tannins, glycosides, flavonoids and saponins was carried out.

2.4 GC-MS examination of methanolic extract

Using a Clarus 500 Perkin Elmer (Auto system XL) Gas Chromatograph coupled to a mass detector Turbo Mass Gold-Perkin Elmer Turbo Mass 5.1 spectrometer, GC-MS analysis of the methanol extract from *C. caesia* was carried out. The identification of compounds from spectral data was based on available mass spectral records.

2.5 Isolation and Characterization of plant extract

The crude powder extract was weighed and dissolved in a small amount of a suitable starting solvent (methanol or chloroform) to create a slurry. A column was filled with silica gel of 200-400 mesh size. The sample mixture was carefully added on top of the silica gel bed. A solvent system was prepared by mixing methanol and chloroform in the desired ratio. The solvent system was poured into the column and allowed to run through by gravity, separating compounds based on polarity. Fractions were periodically analyzed using TLC to identify when the target compounds had eluted. Fractions containing the compounds of

interest, as determined by TLC analysis, were pooled. The analysis employed an Elite1 capillary column with a 0.25mm ID and 1m length [1–17]. In High-Performance Liquid Chromatography (HPLC), a prepared sample is introduced into the system and run through a column filled with a stationary phase. The mobile phase, composed of a solvent or a solvent mixture, propels the sample through the column. These peaks are detected and analyzed to determine and quantified the individual components in the sample.

2.6 Docking and ADMET studies

Docking studies were conducted using Auto Dock 4.2.6 software, and the protein-ligand interactions in 2D were visualized using Discovery Studio Visualizer. The 2D structure of the ligand 3-Bromocamphore-8-sulphonic ammonium salt" was drawn using Chem Sketch. The protein structures, 8HCR (Cryo-EM structure of the *Mycobacterium tuberculosis* cytochrome bcc:aa3 supercomplex and a novel inhibitor targeting subunit cytochrome cI) and 5XFS (Crystal structure of PE8-PPE15 in complex with EspG5 from *M. tuberculosis*), were obtained from the Protein Data Bank (PDB). The 3D receptor was prepared by removing water molecules and co-ligands using PyMOL. Swiss ADME software was employed to evaluate the 3-Bromocamphore-8-sulphonic ammonium salt for any violations of Lipinski's rule of five.

2.7 Anti-Tubercular Activity and MTT Assay

The assessment of compound efficacy against *Mycobacterium tuberculosis* was conducted through the utilization of the microplate Alamar Blue Assay (MABA), a method recognized for its safety and reliability. Notably, this approach employs a thermally stable reagent and exhibits strong agreement with both proportional and BACTEC radiometric techniques. The *Mycobacterium tuberculosis* H37 RV Strain (ATCC No-27294) was employed. The anti-tuberculosis test standard values were as follows: Ethambutol-1.6μg/ml, Isoniazid-1.6μg/ml, Rifampicin-0.8μg/ml, Pyrazinamide-3.125μg/ml, and Streptomycin-0.8μg/ml.

At CDRI-CSIR Lucknow, in accordance with their established methodology, *in vitro* cytotoxicity assays were conducted. These assays focused on investigating the cytotoxic effects of synthesized compounds on *M. tuberculosis* cell lines, specifically Vero cells (ATCC CCL-81), across various dosages. The blue formazan product formation in this assay is a result of the metabolic conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by active cells.

The following formula was used to determine the percentage of cytotoxicity.

% cytotoxicity =100*[1-(X/R1)]

Where X = absorbance of the sample at 540 nm

R1 = absorbance of control at 540 nm.

The Prism software version 4.01 was used to calculate the IC50 values using a nonlinear regression model

3 Results and Discussion

Preliminary phytochemical test for *C. caesia* extract showed positive for steroids, phytosterols, alkaloids, flavonoids, glycosides, tannins, saponins, proteins, terpenoids, phenolics and negative for resins and gum [Table 1]. Previous research also confirmed the presence of phytochemicals of different classes such as flavonoids, tannins, alkaloids, phenolic compounds, saponins and coumarin [18]. GC-MS study revealed the presence of 3-Bromocamphor-8-sulfonic acid ammonium [Table 2]. Atom et al. reported the presence of 16 compounds from petroleum extract and 20 compounds from chloroform extract of *C. caesia* in GC-MS evaluation and the major compounds identified were sesquiterpenes [18].

Table 1. Phytochemical screening of *Curcuma caseia* extract

Chemical tests	Report
Alkaloids	+
Flavonoids	+
Glycosides	+
Phenolics	+
Steroidal compounds	+
Phytosterols	+
Tannins	+
Saponins	+
Resins	-

Continued on next page

Table 1 continued

Proteins	+
Terpenoids	+
Gum	-

+ Present, - Absent

Table 2. Retention time of GC-MS Compounds

Sl.no.	Compound name	Molecular Formula	Molecular Mass(g/mole)	Retention time(min)
1.	3-Bromocamphor-8-sulfonic acid ammonium salt	$C_{10}H_{15}BrO_4S \cdot NH_3$	328	16.625
2.	Nonadecanoic acid	$C_{19}H_{38}O_2$	298	14.214
3.	5-Methyloxazolidine	C_4H_9ON	87	15.024
4.	Hydroxylamine, o-(3methylbutyl)	$C_5H_{13}ON$	103	15.279
5.	2,3-Anhydro-d-galactosan	C_6H_804	144	15.669
6.	Heptanal	$C_7H_{14}O$	114	16.009
7.	Acetic acid	$C_2H_4O_2$	60	16.124
8.	Propanedioic acid	$C_3H_4O_4$	104	18.275
9.	2-Isopropoxyethylamine	$C_5H_{13}ON$	103	19.471
10.	Ethanamine, 2-propoxy-	$C_5H_{13}ON$	103	19.506

3.1 Isolation & Characterization

The desired compound was isolated with column chromatography, and a TLC chamber with a 10% mass/volume concentration was used. The mobile phase consisted of 1 ml of chloroform and 9 ml of methanol, while the stationary phase employed silica gel. Confirmation of the isolated compound was achieved through TLC analysis [Figure 1]. Methanol was used to extract the compound from *C. caesia* and filtered to separate the extract, concentrated, and then characterized it.

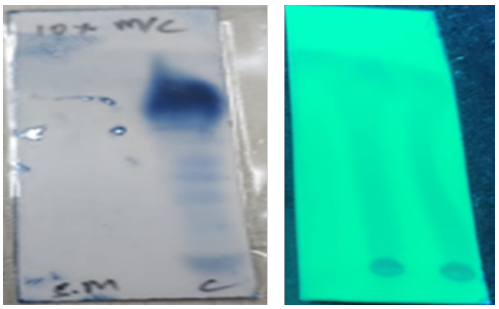

Fig 1. TLC of crude sample and standard

3.2 HPLC

HPLC analysis was performed for quantification of extracted curcumin from turmeric powder and compared with standard. HPLC analysis of the methanolic extract of *C. caesia*, using (+)-3-Bromocamphor-8-Sulfonic acid ammonium salt as a sample and a standard compound, revealed a significant difference in purity. The standard exhibited a purity of 98.027%, while the sample had a purity of 0.744% [Figure 2 & Table 3].

3.3 Docking and ADMET results

Docking studies indicated the binding energies of (+)-3-Bromocamphor-8-Sulfonic Acid Ammonium Salts with a total of six proteins, with 8CHR exhibiting the highest docking score of -7.5, followed by 5XFS with -7.1, 6VJ5 with -7.1, 5BUQ with -6.5, 4KXR with -6.8, and 7Q18 with -6.5. The Swiss ADME software was employed to assess (+)-3-Bromocamphor-8-Sulfonic Acid

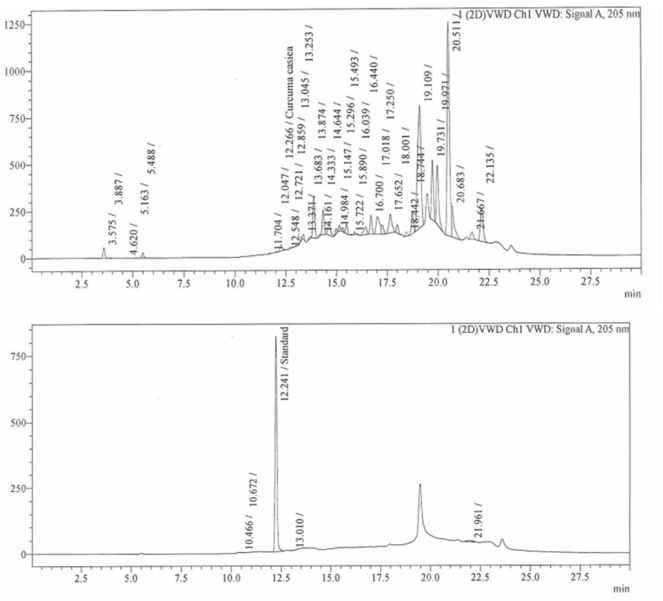

Fig 2. Spectrum of sample and standard

Table 3. Retention time of sample and standard

Peak	Retention time	Height	Area	Compound name	Area %	RRT
8	12.266	34758	264417	Sample	0.744	1.00
3	12.241	814616	5151390	standard	98.027	1.00

Standard - 3-Bromocamphor-8-sulfonic acid ammonium salt; Sample - Methanolic extract of *C. caesia*

Ammonium salts for any potential violations of Lipinski's Rule of Five. The results of the docking study highlighted 8CHR as the protein with the most robust binding affinity for (+)-3-Bromocamphor-8-Sulfonic Acid Ammonium Salts. For a comprehensive evaluation of its drug-likeness, Swiss ADME software should be used to ensure compliance with Lipinski's Rule of Five, a crucial consideration in assessing its potential as a drug candidate [Figure 3].

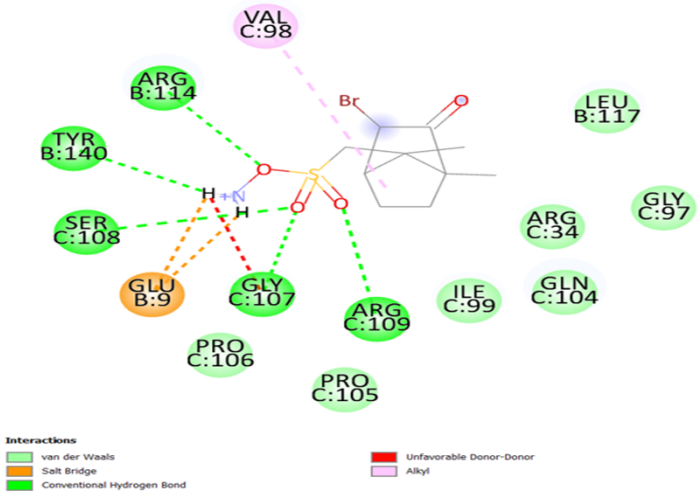

Fig 3. Docking protein (PDB: 5XFS) with ligand

In the study of Atom et al., molecular docking studies were done with four metabolites, namely, beta-elemene, curzerenone, boldenone, and 2-cyclohexen-1-one, 4-ethynyl-4-hydroxy-3, 5, 5-trimethyl with 4DRE and 3UCI. Enoyl-acyl reductase enzyme (InhA) is necessary for cell wall synthesis as it synthesizes mycolic acid a vital component of the mycobacterium cell wall and gyrase type IIA that helps in reducing topological strain in DNA helix during replication. All four metabolites followed Lipinski's rule of five which is consistent with our study. The estimation of the ADMET properties plays a significant role in the early phase of the drug formulation process. The docking study findings revealed that boldenone showed the highest binding energy against both the receptor 4DRE and 3UCI. The most efficient binding was shown by the 3UCI protein with boldenone, which is −8.45 Kcal/mol with three hydrogen bonds and nine hydrophobic interactions [18].

3.4 Anti-Tubercular and MTT Assay

Eight distinct levels (100, 50, 25, 12.5, 6.2, 3.12, 1.6, and 0.8μg/ml) of both the test and standard drug were tested for the potential of anti-tubercular activity. The methanolic extract of *C. caesia* remained sensitive for the *Mycobacterium* strain up to 3.12 μg/ml concentration whereas -3-Bromocamphor-8-sulfonic acid ammonium salt was sensitive up to 6.2 μg/ml concentration as shown in Table 4.

Table 4. Antitubercular activity of the Methanolic extract of *C. caesia* and [(1R) -(endo, anti)] -(+)-3-Bromocamphor-8-sulfonic acid ammonium salt

Sl. No.	Sample	100 μg/ml	50 μg/ml	25 μg/ml	12.5 μg/ml	6.2 μg/ml	3.12 μg/ml	1.6 μg/ml	0.8 μg/ml
1.	S-1	S	S	S	S	S	S	R	R
2.	S-2	S	S	S	S	S	R	R	R

S – Sensitive; R- Resistant

Many studies suggested that sesquiterpene has the property of anti-mycobacterium activity [19] for example β-elemene has the capacity to alter the expression of the dprE1 gene needed for cell wall synthesis and clgR genes regulate cell membrane structure of mycobacterium. The essential oil present in ginger is predominantly composed of monoterpenes as well as sesquiterpenes which demonstrated inhibitory activity against *Mycobacterium tuberculosis* [20]. The presence of sesquiterpene in herbal extract is responsible for anti-mycobacterium activity [18]. In the present study, bicyclic monoterpenes present in the methanolic extract are responsible for anti-tuberculosis activity. In contrast, steroids are also effective in reducing tuberculosis mortality, including pulmonary tuberculosis [21]. Steroids given in combination with antituberculosis drugs showed a decrease in the mortality rate in patients suffering from tuberculosis of the central nervous system [22]. Testosterone and estradiol derivatives have potential anti-mycobacterium activity with IC50 at 10.6 μM. Also, corticosteroids are identified to have a beneficial effect on the persistence of tuberculosis patients [21]. In the present study, the anti-mycobacterium activity of *C. caesia* seems to be due to the presence of 3-Bromocamphor-8-sulfonic acid ammonium salt. *In silico* analysis is an attractive method used by researchers to recognize the interactions among drugs and proteins as it is beneficial for the synthesis of a better drug for a specific pathogen.

The *in vitro* cytotoxic effects of 3-Bromocamphor-8-sulfonic acid ammonium on Vero cells (ATCC CCL-81) were evaluated using the MTT assay. The screening outcomes presented in Table 5 indicate that the standard possesses significant antitubercular activity. The sample as well as doxorubicin displayed IC_{50} values below 10 in the Vero cell lines. It has previously been demonstrated that naturally occurring diterpenoids are cytotoxic to human carcinoma cell lines. [23].

Table 5. IC50 and Selectivity Index of Sample and Standard compounds

Sl. No.	Compound	MIC (μg/ml)	IC50	Selectivity Index
1.	Standard	2.5	10.0	4.0
2.	Sample	1.5	3.5	3.0
3.	Doxorubicin	5	7.3	1.5

4 Conclusion

The phytochemical evaluation of this study reveals the existence of alkaloids, carbohydrates, flavonoids, glycosides, tannins, and saponins in *C. caesia* roots, traditionally utilized for various ailments, reflecting its rich repository of active constituents. Utilizing methanol for extraction led to the identification of these bioactive compounds via phytochemical tests, further confirmed through GC-MS analysis, uncovering multiple bioactive compounds. Docking studies demonstrated a notable

docking score of -7.5 for (+)-3-Bromocamphor-8-Sulfonic Acid Ammonium Salts with protein 5XFS. Isolation and characterization of these compounds added depth to the research, while the low Minimum Inhibitory Concentration in the anti-TB assay suggests potent inhibitory effects on *Mycobacterium tuberculosis*, emphasizing the potential of *C. caesia* as an anti-TB agent. This discovery emphasizes the need for additional research and clinical trials to explore its safety and efficacy to develop novel anti-TB agents against sensitive and drug-resistant strains of *Mycobacterium tuberculosis*.

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