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Chemical Composition, *in Vitro* Antimycobacterial Assay, and *in Silico* Studies to Identify Potential *Mycobacterium tuberculosis* Inhibitors from *Icacinatrichantha* Tubers

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Abstract: *Mycobacterium tuberculosis* is a bacterial parasite microbe that infects approximately 33% of the world's population. This work is aimed at identify potential *Mycobacterium tuberculosis* inhibitors from the ethylacetate/methanol fraction of *Icacinatrichantha* tuber through GC-MS analysis, *in vitro* antimycobacterial assay, and *in silico* studies. Extraction, column chromatograpgy and GC-MS analysis of *Icacinatrichantha* tubers were performed. The antimycobacterial activity of the plant extracts was studied using the *Mycobacterium* TB strains H37Rv, ATCC 27294, and multi-drug resistance strains. The MicroplateAlamar Blue Assay (MABA) test was used to assess the fraction. Nine compounds were identified from the GC-MS spectrum. The fraction's MIC against *M. tuberculosis* MDR-TB and H37RV strain were found to be 3.13±0.00 μg/mL. In terms of efficacy against MDR M. tuberculosis strains and H37Rv, the fraction was very potent. Molecular docking revealed that all the compounds have good binding energies with *M. tuberculosis* protein. All the compounds met Lipinski's Rule of Five requirements (RO5). From RO5 results, the compounds will have reduced rates of attrition in clinical trials and hence a better probability of getting launched. The findings suggest that the compounds found in the ethylacetate/methanol fractionof *I. trichantha* (Oliv) may be developed as a potential tuberculosis drug.

Keywords: *Icacinatrichantha* Oliv,GC-MS, *Mycobacterium tuberculosis*, docking, tubers, *in silico*

Introduction

Tuberculosis is a contagious disease that primarily impacts the lungs and has the potential to be deadly. The bacteria that cause tuberculosis spreads from person to person through tiny droplets released into the air when someone coughs or sneezes. TB infections started to increase in developed nations in 1985, owing primarily to the introduction of HIV, the AIDS-causing virus. HIV impairs an individual's immune system, preventing it from fighting TB bacteria (CDCP, 2020). Due to tighter control methods, tuberculosis rates in the United States started to decline (CDCP, 2020). It still raises some concerns, though. The majority of antituberculosis drugs are ineffective against certain tuberculosis strains. In order to eradicate the illness and prevent antibiotic resistance, people with tuberculosis must take a range of drugs for several months. In order to contain this global health emergency, novel ways to inhibit mycobacterial pathogenesis or strengthen the host response in the face of mycobacteria must be developed.

The search for new lead compounds for the treatment of tuberculosis is progressively shifting toward natural sources. Plants are important components of traditional healing methods and mainstream medicine. Herbal medicine was used before antibiotics, as well as before economic, societal, and religious limitations.

I. trichantha (Icacinaceae) is classified as a perennial shrub that grows in forest regeneration, field crops, and waste areas. *I. trichantha*Oliv's leaves are arranged alternately, simply, and broadly elliptically. The stem is uneven, somewhat wooden, bent in cross-section, and covered in soft brown hairs. It grows from an underground tuber with soft brown hairs as well. The Nigerian Yoruba tribe uses the leaves at chieftaincy coronation (Kadiri*et al*., 2020). The leaves are used by the Igbos to enfold processed oil bean seeds known as "ugba".(Kadiri*et al*., 2020). In Nigeria's rural villages, the herb is commonly used as a drug.[2] The Igbos consider the plant to be an aphrodisiac (Kadiri*et al*., 2020). .Samuel and colleagues claimed that *I. trichantha* sodium arsenate hepatoprotective properties caused genotoxicity, indicating that it may be used as an anti-tumour agent (Kadiri*et al*., 2020). *I. trichantha*is a plant that natives of West Africa use for food and medicine. In animal models, the tuber has a wide range of pharmacological effects due to its high starch content. A series of 19-*nor*-pimarane-type unusual diterpenes has been discovered by chemical analysis (Brian *et al*., 2020). In a concentration-and time-dependent mode, water-based extract from I. trichantha's tuber was discovered to eradicate termites (Chimezie*et al.,* 2021).Phytochemical analysis of *I. trichantha* leaf extract suggested that alkaloids, saponins, phenols, tannins, and fatty acids were present (Chimezie*et al.,* 2021). There have been reports *of I. trichantha*Oliv leaf aqueous extract oral toxicity, both acute and subchronic(Timothy *et al.,* 2018). Both male and female mice showed no mortality from acute or subchronic poisoning. (Timothy *et al.,* 2018).Saponins, alkaloids, steroids, tannins and glycosides were discovered during phytochemical

screening of *I. trichantha* fractions (Ojah and,Kachi, 2020).Antibacterial growth inhibitory effects in the agar diffusion assay of *I.trichantha* fractions have been reported (Ojah and,Kachi, 2020).

Numerous researchers have identified phytocompounds in plants using GC-MS (Kwekowe*et al*., 2021; Ikpeazu*et al*., 2017; Otuokere*et al*., 2016a; Otuokere*et al*., 2016b, Igwe*et al.,* 2016a; Igwe*et al*., 2015; Igwe*et al.,* 2016b; Ahuchaogu*et al*., 2020; Ikpeazu et al., 2020a; Ikpeazu et al., 2020b, Otuokere*et al*., 2016c. Only few documentations exists on the structural formula of the leaves of *I. trichantha.*There is no information on the GC-MS of *I. trichantha*tubers or the molecular docking studies of bioactive phytocompounds. There are no reports on the *in vitro* antimycobacterial assay of the ethylacetate/methanol fraction of *I. trichantha*tubers. To our best understanding, this is the first I. trichantha tubers study employing GC-MS analysis, *in vitro* antimycobacterial assay, molecular docking, drug-likeness and *in silico* toxicity. As a result, the aim of this research is to uncover possible tuberculosis inhibitors from *I.trichantha*ethylacetate/methanol fraction using GC-MS, *in vitro* antimycobacterial assay and molecular docking.

Materials and Methods

Extraction and Column Chromatography

On September 24, 2018, between 2.00 and 5.30 p.m, tubers of *I.trichantha* were harvested in Umudike, Abia, Nigeria. The Taxonomy section of the Michael Okpara University of Agriculture, Umudike, identified the plant and assigned it the herbarium number ICA DALZ 1094. The tubers were peeled and grated after being washed to eliminate grit. Upon air drying for four weeks, the grated tuber was weighed. *I. trichantha* powdered air-dried tubers (520 g) were macerated in 5 L of $CHCl_3$ for 72 hours at room temperature. After filtering, the extract was vacuum-concentrated at 40 °C. Thirty eight (38) g of crude extract were obtained. Hex:$CHCl_3$(100→0), $CHCl_3$:$CH_3COOC_2H_5$(100→0), and $CH_3COOC_2H_5$:MeOH (100→0), were used to elute the $CHCl_3$ extract, using CC in silica gel (150 g). Ninety-one (91) fractions of 125 mL each were obtained. After being separated, column fraction 33, an oil fraction eluted from $CH_3COOC_2H_5$: MeOH (50:50), was analyzed using GC-MS analysis.

GC-MS Evaluation

The test was conducted using a 7890A GC-MS Triple Quad instrument (Agilent Technologies). A precise 1.5 μL of the sample was injected in the split-less mode. The supply and injector were both adjusted to 250°C. The temperature in the oven was 40 °C at first, then steadily increased to 300 °C at a pace of 10 °C/min for a total of 60 minutes. After the run, the temperature was set to 305 °C and sustained for 1 minute. The EI mode of the MS used was 70 eV. From m/z 50 to 650. The individual mass spectral peak values of the fraction's unidentified

phytochemical components were compared to the National Institute of Science and Technology's 2014 database in order to identify them.

Antimycobacterial Activity

The antimycobacterial activity of the plant extracts was studied using the *Mycobacterium* TB strains H37Rv, ATCC 27294, and multi-drug resistance strains of *M. tuberculosis*. The MABA test was used to assess the fractions (Christoph*et al*., 2018). Dimethyl sulfoxide (20 mg/mL) was used to dissolve the sample. In 96-well sterile microplates,A suspension of 100 μL of every mycobacterium was introduced after the sample had been serially diluted (range: 1.00-3.12 μg/mL) in 7H9 broth (Nunc). Plates were cultured for *M. tuberculosis* for five days at 35°C. Every assay was performed twice. Moxifloxacin (0.1 g/mL) and isoniazid (0.06 g/mL) were used as positive controls.

M. tuberculosis protein preparation and identified compounds

The RCSB Protein Databank was used in obtaining *M. tuberculosis* protein (PDB ID: 1W30). ArgusLab 4.0.1 software(Thompson, 2004)was used to extract the water molecules. The structures of the compounds were drawn using ACDLabChemSketch software. The structures of the compounds were converted to PDB using ArgusLab 4.0.1 software (Thompson, 2004)

Molecular docking research

Docking was done using PyRx Virtual Screening Tool (Dallakyan and Olson, 2015). The bond lengths, interaction types, and three-dimensional images of every docked complex were viewed using the PLIP Server (Adasme*et al*., 2021)

The property of drug-likeness

The drug-likeness, Lipinski's RO5 (Lipinski*et al*., 2012) was studied using the Swiss ADME server (SWISSADME, 2022)

In Silico toxicity Study

ProTox-II (ProTox-II, 2022) was utilized to predict the toxicity and lethal dose (LD_{50}) of the compounds.

Results and Discussion

Statistics analyses were applied to the data. Data were presented as the mean±SD. The student unpaired t-test was used to examine the statistical significance of the differences between the various groups. For a $P<0.05$, differences were deemed statistically significant.

Tables and Figures

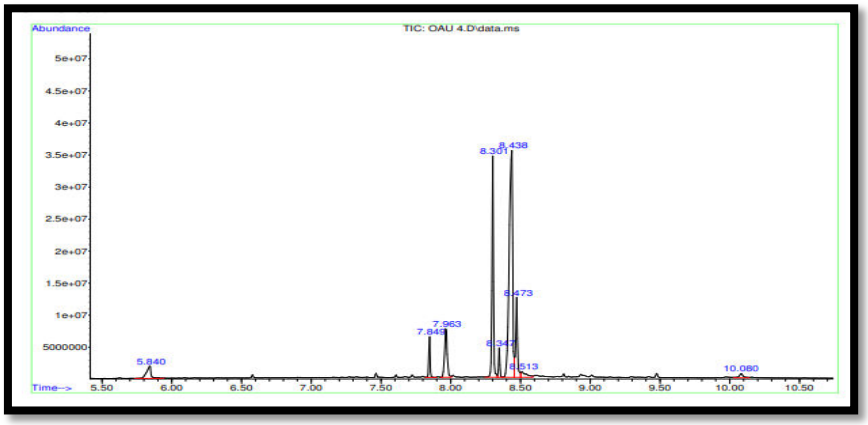


Figure 1: GC chromatogram of the diethylester/methanol fraction of *I. trichantha* tuber.

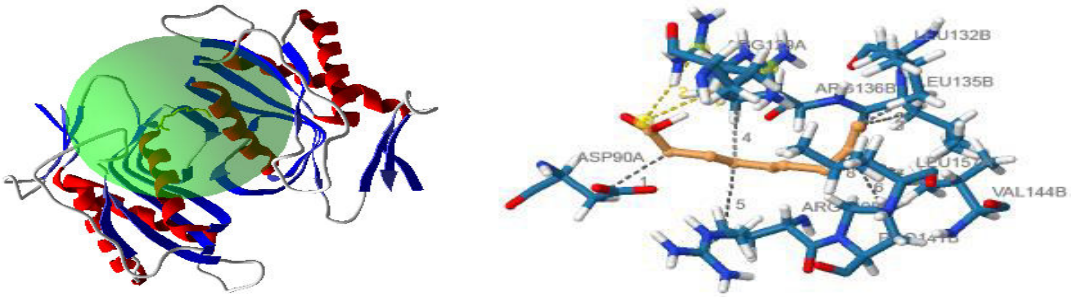
Table 1: Antimycobacterial assay results (MIC) of the ethylacetate/methanol fraction of *I. trichantha* tubers

***M. tuberculosis* strain**	**Fraction (µg/mL)**	**Moxifloxacin (µg/mL)**	**Isoniazid (µg/mL)**
MDR-TB	3.13±0.00 [a]	3.13±0.00 [a]	-
H37RV	3.13±0.00 [a]	-	1.57±0.00 [b]

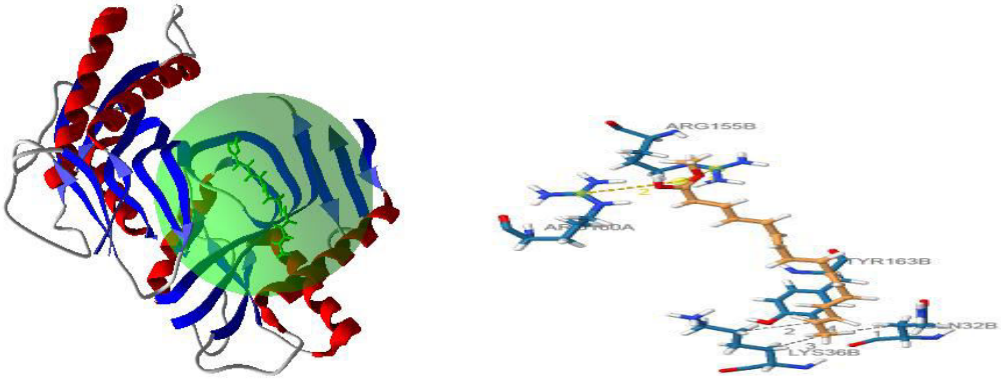
At (P<0.05), means within the row with different superscripts differ significantly, whereas at (P>0.05), there is no significant difference between the means with the same superscript.

Table 2: Phytocompounds present in the GC-MS of the diethylester/methanol fraction of *I. trichantha* tuber.

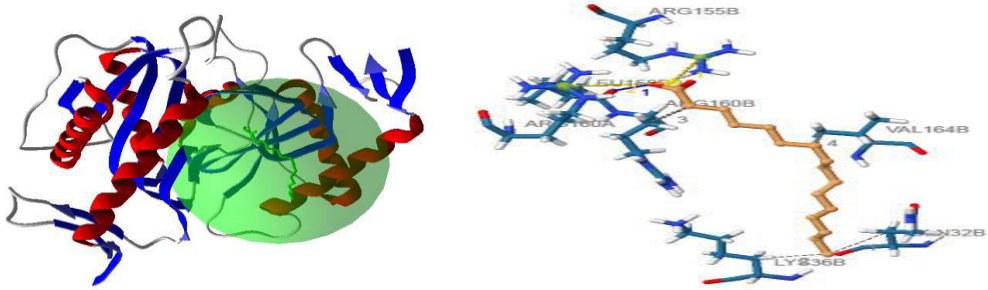
S/No	**Retention Time (mins)**	**Composition (%)**	**Compound**	**Similarity index with NIST Library (%)**
1	5.840	3.57	Nonanoic acid	93
2	7.849	3.45	Methyl ester of hexadecanoic acid	98
3	7.963	8.30	n-Hexadecanoic acid	99
4	8.301	19.74	cis-Vaccenic acid	99
5	8.347	2.22	Methyl stearate	99
6	8.438	50.72	Octadec-9-ynoic acid, DMOX derivative	99
7	8.473	9.05	Octadecanoic acid	99
8	8.513	1.86	Oleic Acid	91
9	10.080	1.10	3-hydroxypropyl ester of oleic acid	90


ASP90A
VAL144B

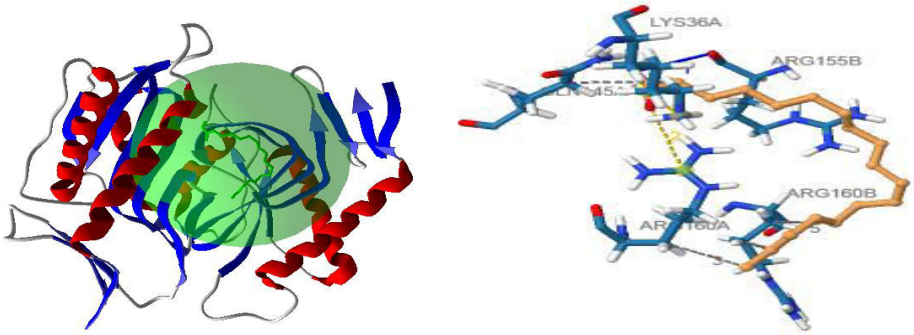
a


ARG155B
TYR163B

b


ARG155B
VAL164B

c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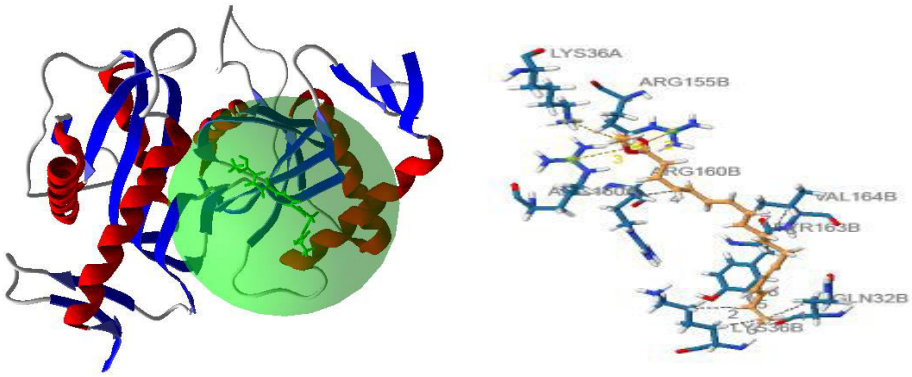
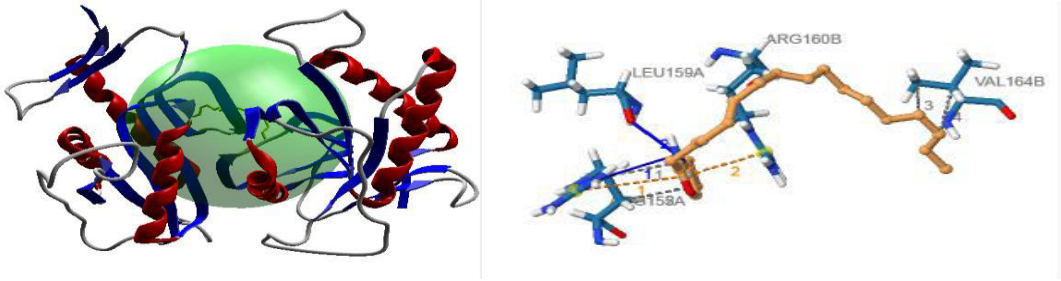
ARG155B
ARG160B

d

e


LYS36A
ARG155B
RG160B
VAL164B
R163B
LN32B
YS36B

f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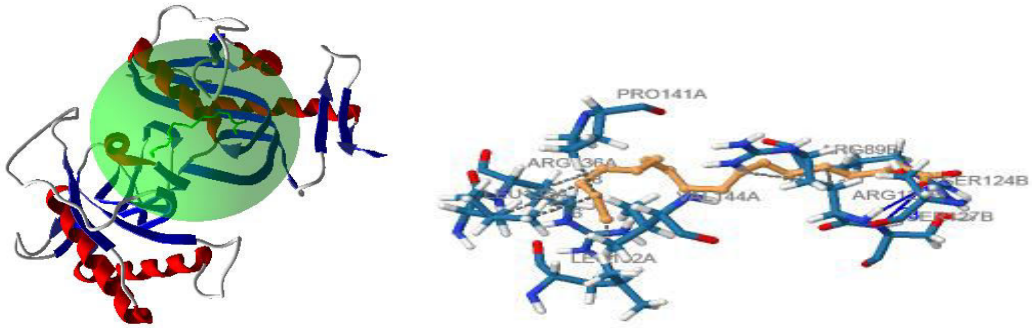
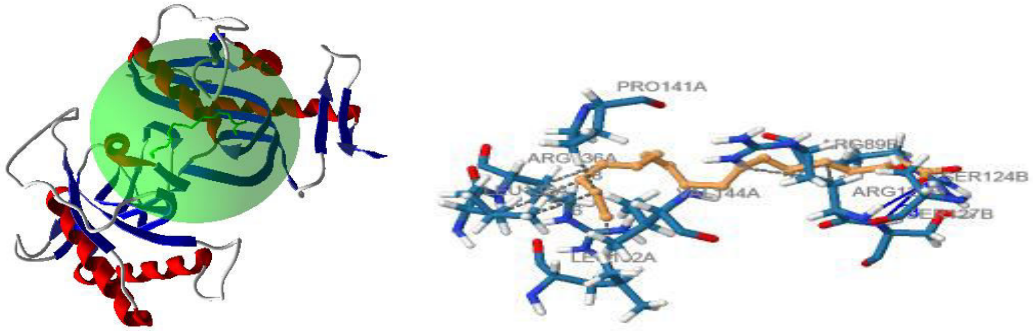
LEU159A
VAL164B

g


PRO141A
ARG138A
ER124B
ARG1

h


PRO141A
ARG138A
ER124B
ARG1

i

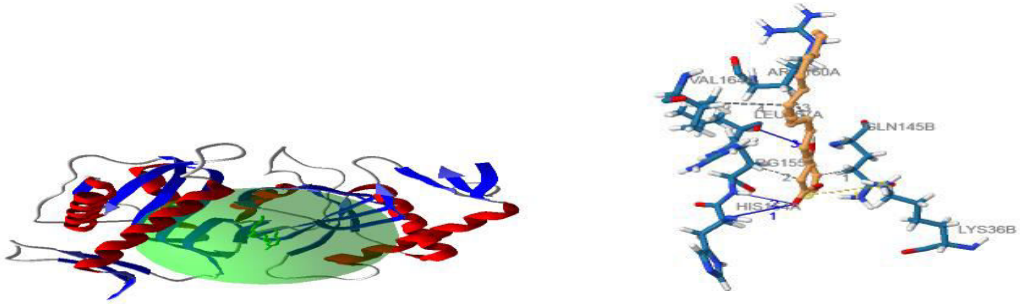

GLN145B
LYS36B

Fig. 2: The bioactive compounds present in the GC-MS of the diethylester/methanol fraction of *I. trichantha* tubers docked with *M. tuberculosis* protein (PDB ID: 1W30) a) Nonanoic acid b) Methyl ester of hexadecanoic acid c) n-Hexadecanoic acid d) cis-Vaccenic acid e) Methyl stearate f) Octadec-9-ynoic acid, DMOX derivative g) Octadecanoic acid h) Octadecanoicacid i) 3-hydroxypropyl ester of oleic acid.

Table 3: Binding affinities and prediction of drug-likeness properties for docked compounds derived from the diethylester/methanol fraction of *I. trichantha.*

Compound	**Binding affinity (kcal/mol)**	**Mol. Weight[1] (g/mol)**	**HB Acceptor[2]**	**HB Donor [3]**	**Lipophilicity[4]**	**Molecular Refractivity [5]**	**Rule of Five [6]**
Nonanoic acid	- 4.8	158.24	2	1	2.82	47.15	0
Methyl ester of hexadecanoic acid	-4.9	270.45	2	0	5.64	85.12	1
n-Hexadecanoic acid	- 5.0	256.42	2	1	4.19	80.80	1
cis-Vaccenic acid	-5.2	282.46	2	1	6.11	89.94	1
Methyl stearate	- 5.6	298.50	2	0	6.42	94.73	1
Octadec-9-ynoic acid, DMOX derivative	-5.6	319.52	2	0	4.86	107.24	1
Octadecanoic acid	- 5.1	282.46	2	1	6.11	89.94	1
Oleic Acid	- 5.1	282.46	2	1	6.11	89.94	1
3-hydroxypropyl ester of oleic acid	- 5.3	340.54	3	1	4.37	105.03	1

Table 4. Toxicity prediction of the phytocompounds from the diethylester/methanol fraction of *I. trichantha* tuber by ProTox-II.

S/No	Compound	Predicted LD_{50}, mg/kg [a]	Predicted Toxicity Class [a]
1	Nonanoic acid	900	4
2	Methyl ester of hexadecanoic acid	5000	5
3	n-Hexadecanoic acid	900	4
4	cis-Vaccenic acid	48	2
5	Methyl stearate	5000	5
6	Octadec-9-ynoic acid, DMOX derivative	2800	5
7	Octadecanoic acid	900	4
8	Oleic Acid	900	4
9	3-hydroxypropyl ester of oleic acid	3520	5

Antimycobacterial assay results of the ethylacetate/methanol fraction of *I. trichantha* tubers is shown in Table 1.According to Table 1, the fraction's MIC against *M. tuberculosis* MDR-TB strain was found to be 3.13±0.00 μg/mL. There was no significant difference (P>0.05) between the MIC of the fraction and moxifloxacin. The fraction's MIC against *M. tuberculosis* H37RV strain was found to be 3.13±0.00 μg/mL. There was significant difference (P<0.05) between the MIC of the fraction and isoniazid. Generally, the fraction was highly active against the H37Rv and MDR *M. tuberculosis* strains. The fraction's antimycobacterial MIC findings compared favorably with the medications used as standard. A number of secondary metabolites from medicinal plants have previously been identified as having *in vitro* action, making them a promising natural source for the identification of anti-TB medicines. The most potent substances reported against *M. tuberculosis* H37Rv at this time are 12-demethylmulticauline isolated from *Salvia multicaulis* (MIC = 0.46 g/mL), micromolide from *Micromelumhirsutum* (MIC = 1.5 g/mL), and (E)-phytol from *Leucasvolkensii* (MIC = 2 g/mL) (Cantrell*et al*., 2001; Newton*et al*., 2002). Sadly, there isn't much information available on how natural substances behave.

GC-MS Analysis

Figure 1 shows GC chromatogram of the diethylester/methanol fraction of *I. trichantha* tuber. GC-MS chromatogram of the fraction of diethylester/methanol of *I. trichantha* tubers revealed a total of 9 peaks corresponding to bioactive

compounds, which were identified by comparing their data to that of known compounds in the NIST library. The phytocompounds present in the GC-MS of diethylester/methanol fraction of *I. trichantha* tuber are listed in Table 2.

Molecular Docking Studies

The binding affinities and 3D interactions are shown in Table 3 and Figure 2 respectively. The docking studies of all the phytocompoundsin the GC-MS of the diethylester/methanol fraction of *I. trichantha* tuber were docked against *M. tuberculosis* (PDB ID: 1W30) The results suggested that methyl stearate andoctadec-9-ynoic acid, DMOX derivative,were the most active of thetested compounds, with a docking score of -5.6 kcal/mol. All the identified phytocompounds were successfully docked to the *M. tuberculosis* protein.Protein residues ASP 90A, LEU 132B, LEU 135B, ARG 136B, ARG 140B, PRO 141B, GLN 32B, LYS 36B, TYR 163B, ARG 160B, VAL 164B, VAL 144B, and LEU 157A were shown to interact hydrophobically. Salt bridges were observed with protein residues LYS 36A, ARG 155B, ARG 160A, ARG 129A and ARG 136B. Protein residues HIS 154A, ARG 155A, LEU 157A, SER 124B, ARG 126B, SER 127B, VAL 164B, and LEU 159B were found to have hydrogen bonds while π-Cation interactions were observed with protein residues ARG 155A and ARG 160B. *I. trichantha*may contain compounds that could be used as drug candidates, according to the docking score results (Table 3).

Drug-likeness analysis

Prediction of drug-likeness for the docked compounds from the diethylester/methanol fraction of *I. trichantha* tuber is shown in Table 3. The online tool SwissADME was used to explore the pharmacokinetics, drug-likeness, and physiochemical characteristics of docked molecules. Except for nonanoic acid, all of the compounds violated lipophilicity requirements, according to RO5. All of the docked compounds, on the other hand, satisfied Lipinski's criteria, which are thought to predict optimal drug-like properties. No compound violated more than one rule. When a drug fails to meet two or more of these criteria, it is classified as non-orally accessible. However, all of the compounds in this investigation had 0 or 1 violation, indicating that they are bioavailable or orally available medications. All the compounds that were docked demonstrated orally active drug-like characteristics, according to Lipinski's criteria. High permeability, great absorption, and bioavailability have been observed for compounds with lower lipophilicity, molecular weight, and hydrogen bond capacity(Li*et al*., 2020; João*et al*, 2021).

Prediction of Toxicity

Table 4 shows the toxicity prediction of the phytocompounds from the diethylester/methanol fraction of *I. trichantha* tuber by ProTox-II. ProTox-II predicted that hexadecanoic acid methyl ester, methyl stearate, octadec-9-ynoic

acid, DMOX and oleic acid, 3-hydroxypropyl ester maybe harmful if consumed ($2000 < LD_{50} \leq 5000$).Nonanoic acid, n-hexadecanoic acid, octadecanoic acid will be harmful if consumed ($300 < LD_{50} \leq 2000$) while cis-vaccenic acid is deadly if consumed ($5 < LD_{50} \leq 50$). To reduce the number of test substances and test animals used in scientific experiments, predicted toxicity *in silico* is carried out before *in vitro* and *in vivo* testing (Varsha and Jhinuk, 2021; Supandi and Merdekawati, 2018).

Conclusion

The diethylester/methanol fraction of *I. trichantha* tuber was studied using GC-MS.The tested H37Rv and MDR M. tuberculosis strains were both greatly inhibited by the fraction. An excellent affinity for *Mycobacterium tuberculosis* was shown by docking experiments. The molecular docking results suggest that *I. trichantha*tuber may be a promising natural *M. tuberculosis* inhibitor. RO5 was met by every identified compound. According to this, there would be less attrition during clinical trials for the identified *I. trichantha*(Oliv) compounds, increasing the likelihood that they would be launched.

Conflict of interest

There is no conflict of interest

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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