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Antimycobacterial and Gas Chromatography-Mass Spectrometry Profiles of *Cassia auriculata, Hallea stipulosa, Euphorbia nyikae,* and *Albizia anthelmintica*

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ABSTRACT

Cassia auriculata (Senna auriculata), Hallea stipulosa, and *Albizia anthelmintica* are among the sources of herbal remedies used to treat tuberculosis and its related symptoms. However, the literature has limited information on the anti-mycobacterial properties of various parts of these plants. This study assessed different parts of *C. auriculata*, *H. stipulosa*, *E. nyikae*, and *A. anthelmintica* for anti-mycobacterial activity and the phytochemical profiles of the active extracts. Dry powdered plant materials were soaked in 95% ethanol and boiled in distilled water for ethanolic and aqueous extractions, respectively. Extracts were dried by vacuum rotary evaporation and freeze-drying. Antimycobacterial activity against *Mycobacterium indicus Pranii* (MIP) and *Mycobacterium madagascariense* (MM) was assessed by the microdilution method and data presented as mean ± standard deviations. Phytochemical analysis of the active extracts was done using Gas Chromatography coupled with Mass Spectrometry (GC-MS). The results revealed that *C. auriculata* aqueous and ethanolic extracts exhibited anti-mycobacterial properties against MM and MIP while *H. stipulosa*, *E. nyikae*, and *A. anthelmintica* extracts were inactive. The ethanolic extracts of *C. auriculata* leaves, flowers, and stem wood showed the highest activity (MIC = 156.25 µg/mL) against MM. The GC-MS showed seven most abundant compounds in different extracts of *C. auriculata* including 2,4-dimethylheptane (1), caryophyllene (2), 7-isopropyl-1,4-dimethyl-1R,2,3,3aS,6,8aS-hexahydroazulene (3), resorcinol (4), α.-methylmannofuranoside (5), 3-*O*-methyl-D-glucose (6), and hexatriacontane (7). The activity of caryophyllene and resorcinol against *M. tuberculosis* has been previously reported. Therefore, the findings of anti-mycobacterial and phytochemical profiles obtained from this study support the traditional use of *C. auriculata* to manage tuberculosis.

***Keywords*:** Antimycobacterial, Phytochemical, *Cassia auriculata*, *Hallea stipulosa*, *Euphorbia nyikae*, *Albizia anthelmintica*

Introduction

Tuberculosis (TB) is a disease resulting from infections by Mycobacteria, mainly by *Mycobacterium tuberculosis* (*Mtb*). Adult people are the most affected by TB, contributing about ninety percent of all developing the disease.[1] About 10.6 million individuals were infected with TB and 1.3 million deaths occurred in 2022.[2] This alarming death toll indicates the persistent global health threat posed by TB, especially in regions with limited access to healthcare.

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Despite being a curable disease, the rise in drug-resistant TB has impacted the treatment outcome, and the selection of drug options is limited by the existence of *Mtb* strains which are resistant to anti-TB medications.[3] Therefore, there is a need to constantly identify chemical groups or new molecules with anti-mycobacterial activity for future control of TB.

Screening for the antimycobacterial activity of extracts from medicinal plants traditionally used to manage TB-related symptoms provides an alternative starting point for identifying new active molecules against *Mtb* strains. Modern science is increasingly turning its attention to these remedies, exploring their potential to yield new treatments for TB, especially in the face of a rising number of drug-resistant strains.[4]

The literature shows that medicinal plants are among the various options communities use to manage TB and its related symptoms in many parts of Africa and Asia.[5,6] Traditional healers in these regions utilize a variety of plants believed to possess therapeutic properties,[7,8] and several bioactive compounds with anti-tubercular activity have been documented from parts of different plant species.[9] *C. auriculata (Senna auriculata)*, *H. stipulosa*, and *A. anthelmintica* are among medicinal plants traditionally used in India, Uganda, and Kenya, respectively for managing TB- and its related symptoms.[10,11,12] However, there is limited information on the antimycobacterial

properties of different parts of these plants. This study presents the anti-mycobacterial activity of ethanolic and aqueous extracts from the different parts of *C. auriculata, H. stipulosa, E. nyikae*, and *A. anthelmintica* and the phytochemical profiles of the active extracts.

Materials and Methods

Collection of Plant materials

Roots, stem bark, stem wood, leaves, pods, and flowers of *C. auriculata* L (*Senna auriculata*), seeds of *E. nyikae* Pax ex Engl., and leaves of *A. anthelmintica* (A. Rich.) Brongn were collected from Mbala Village (GPS: 6º36'52.89696" S and 38º31'33.17268" E) in Chalinze district council in August 2022. Roots, stem bark, and leaves of *H. stipulosa* (DC.) J.-F. Leroy were collected from Kilimanjaro (GPS: Location 37M0326499, U.T.M 99373008, Altitude 1774 m) in April 2023. Herbarium voucher specimens with collection numbers SH 1507, SH 1508, SH 1510, and SH 1511 for *E. nyikae, C. auriculata, A. anthelmintica,* and *H. stipulosa*, respectively were kept in the Herbarium at the Institute of Traditional Medicine. All plant parts were dried and ground to coarse powders (sieve size 4 mm).

Chemicals and Reagents

Dimethyl sulfoxide (Carlo erba®, France), ciprofloxacin, glycerol (Carlo erba®, France), ethanol, Middlebrook 7H9 broth base (Sigma Aldrich), p-Iodonitrotetrazolium chloride (Sigma-Aldrich, US), and 96-well plates (Biologix Europe GmbH) were purchased from local suppliers in Tanzania.

Mycobacteria

Mycobacterium madagascariense (MM) and *Mycobacterium indicus Pranii* (MIP) laboratory strains with reference numbers DSM 44641 and DSM 45239, respectively were obtained from DSMZ, Braunschweig, Germany.

Ethanol Extraction

E. nyikae seeds, *A. anthelmintica* leaves, *H. stipulosa* roots, stem bark and leaves, *C. auriculata* roots, stem bark, stem wood, leaves, and pods, and fresh flowers of *C. auriculata* were extracted by maceration of dry powdered plant materials in ethanol at room temperature. Each plant material (500 g) was soaked separately in 2.5 L of 95% ethanol and kept at room temperature for 48 hours with occasional agitation. After 48 hours, solid materials were separated by filtration through cotton wool. Ethanol was removed by rotary evaporation at reduced pressure and temperature (50 ºC) using HAHNVAPOR (HAHNSHIN S&T Co. Ltd, South Korea). Freeze-drying of the semi-solid extracts at low temperature (-80 ºC) and pressures (24 Pa) using Virtis SP Scientific bench top freeze drier (Genevac Ltd, Ipswich, England) was done to obtain dry extracts.

Aqueous Extraction

Dry powdered plant material (100 g) of different parts of *C. auriculata, E. nyikae, H. stipulosa,* and *A. anthelmintica* were kept separately in flat-bottomed flasks. After that, 500 mL distilled water was added followed by heating using a hot plate (Stuart Scientific, UK) to boil. The materials were left in boiling water for 5 minutes. After 5 minutes, the mixtures were allowed to cool, filtered through cotton wool, and the aliquots of extracts in 100 mL beakers were frozen at -20ºC. Dry aqueous extracts were obtained after freeze-drying the frozen aqueous extracts.

Determination of anti-mycobacterial activity

The anti-mycobacterial activity of *C. auriculata, E. nyikae, H. stipulosa*, and *A. anthelmintica* extracts was assessed by determining their minimum inhibitory concentration (MIC) using the microdilution method.[13] The MM and MIP mycobacteria species were maintained in sterile Middlebrook 7H9 broth medium with 0.4% glycerol supplement. The incubation temperature was 37 ºC for MIP and 31 ºC for MM for 5 days before testing. Stock solution (20 mg/mL) of each extract was made by dissolving dry extracts in 0.25 mL dimethyl sulfoxide (DMSO) and 0.75 mL distilled water. Working solutions (5 mg/mL) were prepared by diluting the stock solutions with sterile Middlebrook 7H9 broth.

Preparation of mycobacterial suspension (approximately 1.2 x 10^8 CFU/mL) and serial dilution of extracts in 96-well plates were done as described previously.[14] Wells with bacteria suspension alone without extracts were used as growth controls, wells with bacterial suspension and DMSO were used as solvent controls (negative control), and wells with ciprofloxacin were included as positive controls. Inhibition of mycobacterial growth was determined after 24 hours of incubation with extracts at 37 ºC and 31 ºC for MIP and MM, respectively, followed by incubation with 40 µL of p-iodonitrotetrazolium chloride (0.02%) for 1 hr. The lowest concentration of each extract with no visible mycobacterial growth (no pink colour) was recorded as the MIC.

Determination of Phytochemical profiles

The GC- MS analysis of the ethanolic and aqueous extracts from *C. auriculata* leaves, flowers, pods, roots, stem bark, and stem wood was performed in India at the Common Facility Center USIC, Shivaji University, Kolhapur, as explained before by Moyo et al.[15] Briefly, all extracts were appropriately diluted in methanol. A precise volume of 1.0 µL of the diluted extracts was introduced into the GC-MS equipment (Shimadzu, Japan, model TQ8050 plus with HS20). The capillary column used for separation was 30 meters long with 0.25 mm internal diameter and a film thickness of 0.25 µm composed of HP-5MS (5% diphenyl) dimethylpolysiloxane. Temperature of the ion source was configured at 200 °C and the electron ionization (EI) at 70 eV. The oven temperature started at 80°C during the first 2 minutes, then increased at 3.5 °C per minute to 250 °C, totaling a run time of 53.33 minutes for the GC. The sample was heated to 300 °C, with a split ratio set to 50:5. The spectral data was acquired through EI mode and the mass analyzer was configured to scan the mass from 40 to 400 atomic mass units (amu) over 5 seconds. The extract components were identified by correlating retention time and mass spectra data with those found in the NIST Mass Spectral Library.[16,17]

Results and Discussion

Anti-mycobacterial activity

The results of the anti-mycobacterial assay showed that different parts of *C. auriculata* ethanolic and aqueous extracts exhibited anti-mycobacterial activity against MM and MIP. Ethanolic extracts of *C. auriculata* leaves, flowers, and stem wood showed the highest activity with a MIC value of 156.25 µg/mL against MM. Generally, the MM was more sensitive to inhibition by *C. auriculata* extracts than MIP species, except the stem bark ethanol extract which demonstrated the same activity against both, MM and MIP at 312.5 µg/mL. Furthermore, the results revealed that aqueous and ethanol extracts of *A. anthelmintica* leaves, *E. nyikae* seeds, and *H. stipulosa* roots, stem bark, and leaves were inactive against MM and MIP (Table 1). Although extracts from *H. stipulosa* and *A. anthelmintica* were not active against MM and MIP *Mycobacteria*, these plants remain important sources of herbal remedies for treating infectious and non-infectious diseases.[18,19,20] The stem bark of *A. anthelmintica* is used traditionally in Kenya to treat tuberculosis and the methanol extract of this plant was previously reported to inhibit the growth of both, slow-growing (*M. kansasii,* and *Mtb)* and fast-growing (*M. smegmatis* and *M. fortuitum*) *Mycobacteria* species.[12] The differences in the anti-mycobacterial properties of *A. anthelmintica* leaf extract reported in this study and stem bark extract reported previously may be due to the variation in the bioactive phytochemicals present in the two plant parts and the difference in susceptibility of the *Mycobacteria* species used in the two studies.

Ethnomedical data informs that all important parts of *C. auriculata* are utilized by various communities in tropical Asia and Africa for their medicinal values. The leaves, flowers, pods, roots, and stem bark are used to manage conditions such as diabetes, venereal diseases, dysentery, blood stool, constipation, and aphrodisiac.[21, 22] Among the plant parts included in this study, only *C. auriculata* leaves and *H. stipulosa* roots were reported by the Traditional Health Practitioners for

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the treatment of tuberculosis and its related symptoms.[10,11] Although there was limited information on the anti-mycobacterial properties of *C. auriculata*, the extracts of *C. sophera*, a plant from the same genus, exhibited antimycobacterial properties against *M. smegmatis* and *Mtb*.[23] Also, *in vivo* pre-clinical studies revealed the potential of *C. auriculata* to protect liver damage caused by anti-TB medications. The aqueous leaf extract inhibited rifampicin-induced liver damage,[24] while methanolic root extract protected the liver from damage by isoniazid, rifampicin, and pyrazinamide anti-TB drugs combination.[25]

Table 1: Anti-mycobacterial activity of *C. auriculata*, *H. stipulosa*, *E. nyikae*, and *A. anthelmintica* against MM and MIP

Plant species	Plant Part	Extract type	MIC (µg/mL)	
			***Mycobacterium madagascariense* (MM)**	***Mycobacterium indicus* Pranii (MIP)**
C. auriculata	F	Ethanol	156.25 ± 00	234.375 ± 45
	L	Ethanol	156.25 ± 00	390.63 ± 135.32
		Aqueous	234.38 ± 90.21	390.63 ± 135.32
	SB	Ethanol	312.5 ± 00	312.5 ± 00
		Aqueous	312.5 ± 00	625.0 ± 00
	SW	Ethanol	156.25 ± 00	312.5 ± 00
		Aqueous	625.0 ± 00	>1250
	R	Ethanol	312.5 ± 00	312.5 ± 00
		Aqueous	>1250	>1250
	P	Ethanol	937.5 ± 360.84	312.5 ± 00
		Aqueous	>1250	>1250
H. stipulosa	R	Ethanol	>1250	>1250
		Aqueous	>1250	>1250
	SB	Ethanol	>1250	>1250
		Aqueous	>1250	>1250
	L	Ethanol	>1250	>1250
		Aqueous	>1250	>1250
A. anthelmintica	L	Ethanol	>1250	>1250
		Aqueous	>1250	>1250
E. nyikae	S	Ethanol	>1250	>1250
Ciprofloxacin drug (Positive control)			0.7813 ± 00	0.121 ± 001

Key: MIC = minimum inhibitory concentration (mean ± standard deviation); F = fruits; L = leaves; SB = stem bark; SW = stem wood; R = root; P = pods; S = seeds. Each value represents a mean of extract tested in duplicate in two experiments.

Phytochemical profiles

The GC-MS profiles of the aqueous and ethanolic extracts of flowers, pods, leaves, stem bark, stem wood and roots of *C. auriculata* showed the presence of 26 compounds that can vaporize at or below 260 ^{0}C (Table 2). The most abundant compounds found in more than two extracts and at higher percentages are shown in Figure 1. These are 2,4-dimethylheptane (1), caryophyllene (2), 7-isopropyl-1,4-dimethyl-1R,2,3,3aS,6,8aS-hexahydroazulene (3), resorcinol (4), α.-methylmannofuranoside (5), 3-*O*-methyl-D-glucose (6) and hexatriacontane (7). Figure 2 shows the GC-MS chromatograms for each extract of the plant parts. Caryophyllene was previously found to be a major component of *Premna odorata* volatile oils which prevented the growth of *Mtb* with IC_{50} below 1.5 µg/ml.[26] α.-Methylmannofuranoside was found to enhance anti-TB drug release inside the cells when it was assembled into nanoparticles for targeting macrophages in anti-tuberculosis inhalation therapy.[27] Resorcinol, a compound identified in ethanol extract of *C. auriculata* leaves, roots, and stem wood was previously isolated from *Ardisia gigantifolia* and demonstrated significant activity against *Mtb* $H_{37}R_V$ in MABA and LORA assays.[28] Therefore, the observed anti-mycobacterial properties of *C. auriculata* are attributed to the availability of various bioactive phytochemicals in different plant parts.

Table 2: GC-MS Results for Phytochemical Compositions of different parts of *C. auriculata*

S/N	RT	Compound	Relative Percentage in Extract										
			CAF-E	CAL-E	CAL-W	CAP-E	CAP-W	CAR-E	CAR-W	CASB-E	CASB-W	CASW-E	CASW-W
1	3.3	Tetramethyl silicate									100.0		
2	3.5	4-Penten-2-ol			59.2								
3	4.2	2,4-Dimethylheptane	48.1				69.5		100.0				
4	11.5	2-butenylmethoxymethylphenyl silane				0.34							
5	17.0	2,3,5,8-Tetramethyldecane					30.5						
6	20.9	Caryophyllene			22.3					8.8			13.1
7	21.5	7-Isopropyl-1,4-dimethyl-1R,2,3,3aS,6,8aS-hexahydroazulene			18.5					7.4			33.5
8	22.5	1aαR,2,3,5,6,7α,7aβ,7bα-octahydro-1,1,4,7-tetramethyl-1H-Cycloprop[e]azulene											7.53

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9	22.8	Resorcinol		18.9			46.1			10.9		
10	23.1	1α,2,4aβ,5,8,8aα-hexahydro-4,7-dimethyl-1-(1-methylethyl) naphthalene						1.95				
11	23.2	1-Isopropyl-4,7-dimethyl-1,2,3,5,6,8a-hexahydronaphthalene										8.2
12	23.3	1α,2,3,4,4aβ,5,6,8aα-octahydro-7-methyl-4-methylene-1-(1-methylethyl) naphthalene						3.7				11.0
13	26.5	τ-Cadinol						7.1				26.6
14	29.3	α.-Methylmannofuranoside				90.5	47.7					
15	29.4	3-*O*-Methyl-D-glucose								76.0		
16	31.0	Neophytadiene		3.1		1.5		5.0				
17	32.0	3,7,11,15-Tetramethyl-2-hexadecen-1-ol		0.9		0.2						
18	34.84	Ethyl 15-methyl-hexadecanoate		3.6								
19	34.85	Ethyl hexadecanoate				1.9						
20	34.9	Ethyl 13-methyl-tetradecanoate	51.9									
21	38.3	Ethyl Linoleate				4.0						
22	42.6	Hexatriacontane		49.0		1.6		66.0				
23	44.3	Eicosane		1.3								
24	44.9	Di-n-octyl phthalate		3.1								
25	48.0	Tetrapentacontane		19.9						13.1		
26	48.9	11-Methyltricosane					6.2					

KEY: RT = Retention Time; CAF-E = *C. auriculata* flowers ethanol extract; CAL-E = *C. auriculata* leaves ethanol extract; CAL-W = *C. auriculata* leaves aqueous extract; CAP-E = *C. auriculata* pods ethanol extract; CAP-W = C. auriculata pods aqueous extract; CAR-E = *C. auriculata* roots ethanol extract; CAR-W = *C. auriculata* roots aqueous extract; CASB-E = *C. auriculata* stem bark ethanol extract; CASB-W = *C. auriculata* stem bark aqueous extract; CASW-E = *C. auriculata* stem wood ethanol extract; CASW-W = *C. auriculata* stem wood aqueous extract.

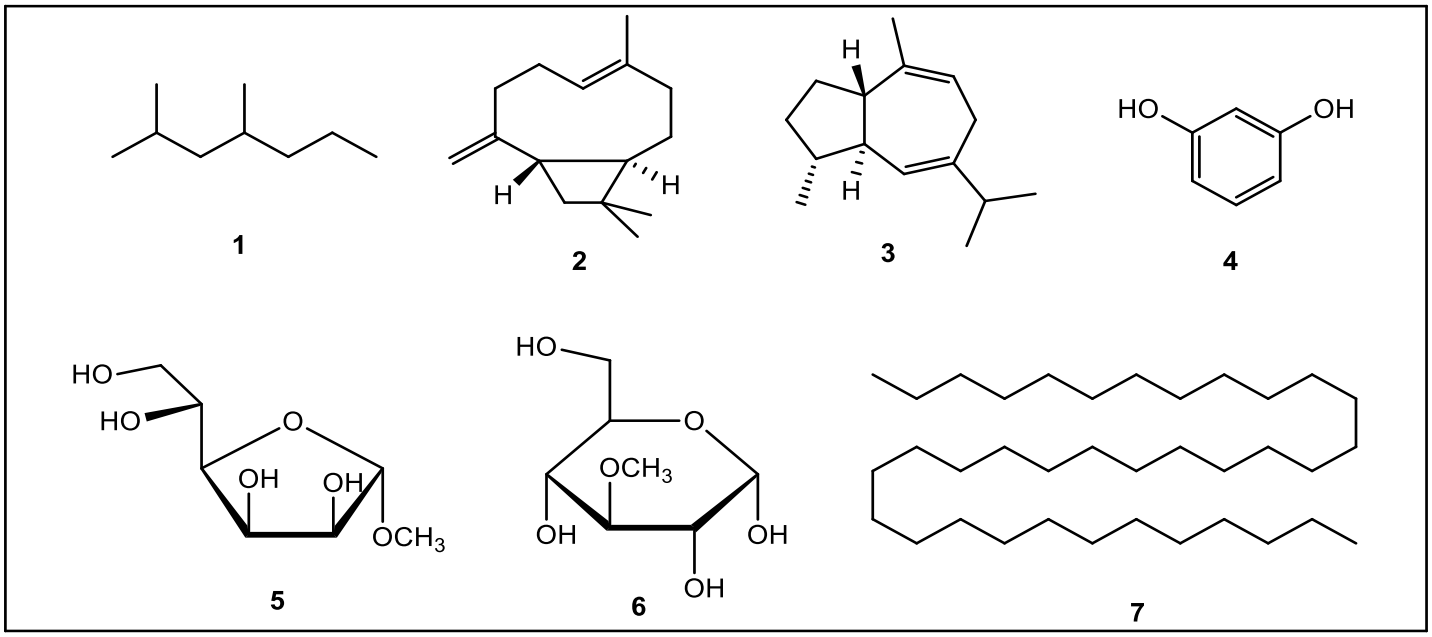

Figure 1: Major GC-MS chemical components of *C. auriculata*. 2,4-dimethylheptane (1), caryophyllene (2), 7-isopropyl-1,4-dimethyl-1R,2,3,3aS,6,8aS-hexahydroazulene (3), resorcinol (4), α.-methylmannofuranoside (5), 3-O-methyl-D-glucose (6), and hexatriacontane (7).

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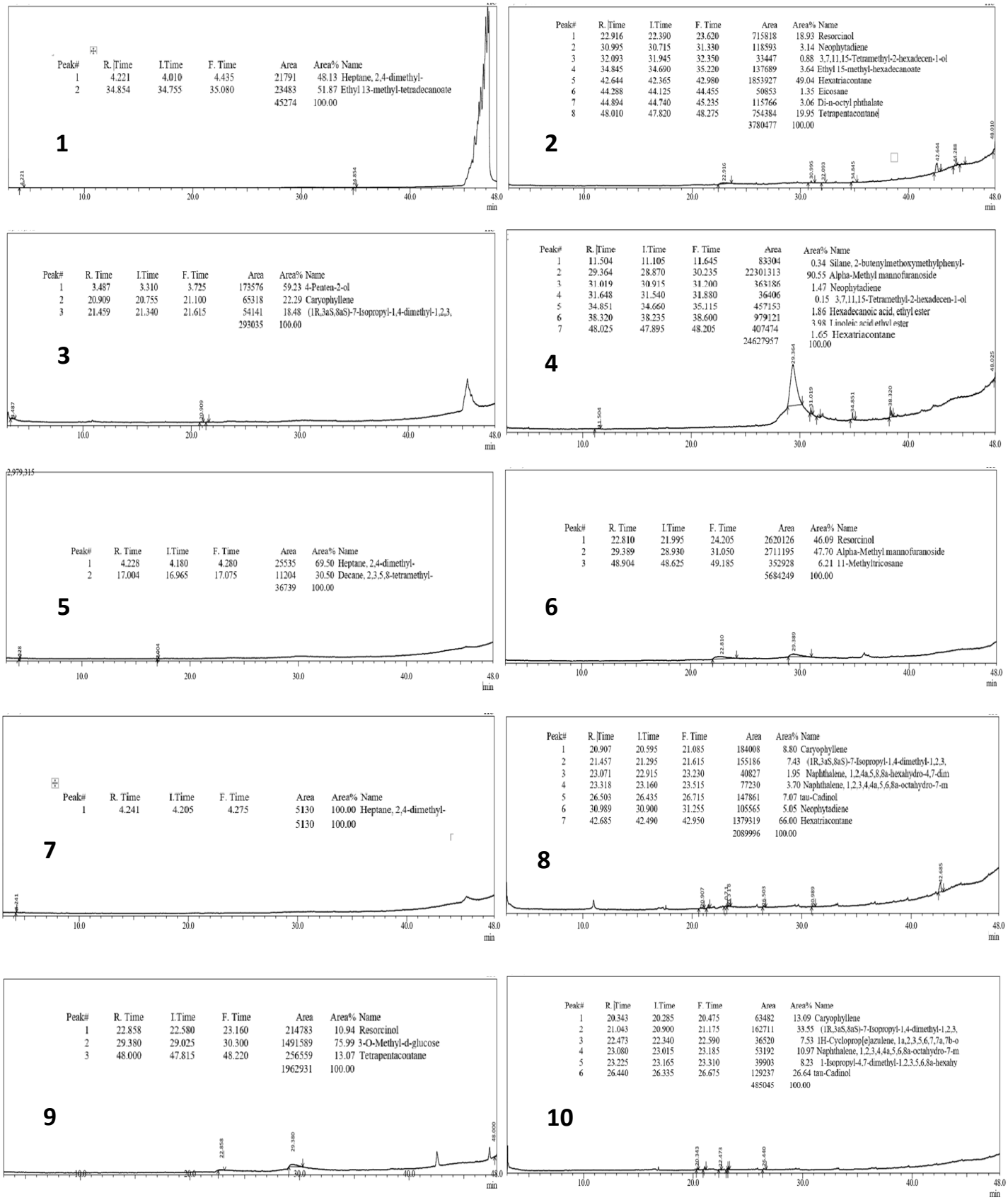

Figure 2: GC chromatogram profiles for *C. auriculata* flowers ethanol extract (**1**); *C. auriculata* leaves ethanol extract (**2**); *C. auriculata* leaves aqueous extract (**3**); *C. auriculata* pods ethanol extract (**4**); *C. auriculata* pods aqueous extract (**5**); *C. auriculata* roots ethanol extract (**6**); *C. auriculata* roots aqueous extract (**7**); *C. auriculata* stem bark ethanol extract (**8**); *C. auriculata* stem wood ethanol extract (**9**); *C. auriculata* stem wood aqueous extract (**10**)

Conclusion

Ethanol and aqueous extracts of *C. auriculata* were active against MM and MIP while *H. stipulosa*, *E. nyikae*, and *A. anthelmintica* extracts were inactive. The anti-mycobacterial and phytochemical profile support traditional uses of *C. auriculata* in managing tuberculosis. Further research is recommended to isolate the active compounds and assess them against *M. tuberculosis.*

Conflict of Interest

The authors declare 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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