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In vitro antiplasmodial activity and cytotoxicity of three *Ziziphus* (Rhamnaceae) species from South Africa

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ABSTRACT

Ethnopharmacological relevance: The Zulu, Swazi, Tsonga, and Venda people of South Africa utilize various parts of *Ziziphus mucronata* Willd., *Z. rivularis* Codd., and *Z. zeyheriana* Sond. to treat different ailments, including malaria. However, despite their use in traditional medicine, *Z. rivularis* and *Z. zeyheriana* remain underexplored for their antiplasmodial activity and cytotoxicity.

Aim of the study: This study assessed the *in vitro* antiplasmodial activity and cytotoxicity of *Z. mucronata*, *Z. rivularis,* and *Z. zeyheriana*. It further identified antiplasmodial constituents using ^{1}H NMR-based metabolomics and GC-MS analyses.

Materials and methods: Ground stem bark, leaf, and root bark samples from each plant species were weighed separately at 40 g before sequential extraction using *n*-hexane, dichloromethane, ethyl acetate, a mixture of ethyl acetate and methanol (1:1; *v/v*), and methanol. Each extraction was performed three times before concentrating the resulting extracts by evaporating the excess organic solvent using a rotary evaporator (Buchi, R-200, Switzerland). Decoctions were also prepared to replicate the traditional preparation method for comparative analysis. Forty-eight successive extracts were obtained and subjected to [^{3}H]hypoxanthine incorporation assay using *P. falciparum* NF54 and cytotoxicity using rat skeletal (L6) myoblast cells. Furthermore, ^{1}H NMR-based metabolomics was used to identify classes of compounds associated with the observed antiplasmodial activity, while GC-MS was employed to identify specific phytoconstituents potentially contributing to this activity.

Results: Five of the 48 tested extracts exhibited high antiplasmodial activity (IC_{50} < 5 μg/ml), while 12 and 17 extracts demonstrated promising (5 μg/ml < IC_{50} ≤ 20 μg/ml) and moderate (20 μg/ml < IC_{50} ≤ 50 μg/ml) activity, respectively. The remaining extracts were inactive (IC_{50} > 50 μg/ml). Notably, the dichloromethane stem bark extract of *Z. mucronata* and the ethyl acetate root extract of *Z. zeyheriana* had the highest antiplasmodial activity, with IC_{50} values of 3.04 and 3.6 μg/ml, respectively. Only the dichloromethane and ethyl acetate stem bark extracts of *Z. zeyheriana* exhibited selectivity, with indices of 10 and 12, respectively. Principal Component Analysis (PCA) did not discriminate the training set based on the observed antiplasmodial activity. However, upon applying the Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA), the samples clustered according to the observed antiplasmodial activity, with R^2 and Q^2 values of 0.8 and 0.7, respectively. Statistically, the OPLS-DA model was significant, with a *P*-value of 0.05. Antiplasmodial activity is linked to aliphatic, allylic, methyl ketone, and carboxylic acid-based classes of constituents. Further GC-MS analysis revealed lupeol, palmitic acid, and friedelin as contributors to the observed antiplasmodial activity in *Z. mucronata*, *Z. rivularis,* and *Z. zeyheriana*.

Conclusion: The study confirmed the significant antiplasmodial activity of *Z. mucronata,* and for the first time, it reported the antiplasmodial activity of *Z. rivularis* and *Z. zeyheriana*. It further demonstrated that the tested samples have no apparent cytotoxicity. The integration of ^{1}H NMR-based metabolomics and GC-MS analysis allowed for the identification of bioactive classes of compounds and the specific constituents contributing to the observed antiplasmodial activity. Lupeol, palmitic acid, and friedelin, previously recognized for their

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antiplasmodial activity, are partly attributed to the observed antiplasmodial activity in *Z. rivularis, Z. zeyheriana*, and *Z. mucronata*.

1. Introduction

Malaria remains a significant global health concern, with 249 million cases and 608,000 deaths reported in 2022 (WHO, 2024). Despite the availability of effective chemotherapeutics, the burden of malaria persists, particularly in sub-Saharan Africa (Lawal et al., 2024). Consequently, antiplasmodial chemotherapeutics continue to be the go-to treatment for *Plasmodium* parasites (Abumsimir and Al-Qaisi, 2023). More than 80 % of currently prescribed drugs are either sourced directly or indirectly from botanical species (Atanasov et al., 2021; Tajbakhsh et al., 2021). Higher plants are rich in secondary metabolites that can be further explored to discover novel antiplasmodial agents. However, effective screening methods for phytoconstituents are needed to address the research limitation that, out of the myriad of phytoconstituents, only a small fraction have been studied (Bitwell et al., 2023). Fortunately, advanced screening techniques such as ^{1}H NMR-based metabolomics and GC-MS expedite the identification of novel effective leads from plants (Najmi et al., 2022; Shi and Zhang, 2021).

The *Ziziphus* (Rhamnaceae) genus is renowned for its medicinal properties in treating diverse human and animal ailments (Ezzouhra et al., 2020). South Africa has three indigenous *Ziziphus* species: *Z. mucronata* Willd., *Z. rivularis* Codd, and *Z. zeyheriana* Sond. While *Z. mucronata* has undergone extensive research, *Z. rivularis* and *Z. zeyheriana* have received less attention due to their infrequent inclusion in various cultural *materia medica*. The Zulu, Swazi, and Vha-Venda communities in South Africa utilize the roots, stems, and leaves of *Z. mucronata* to treat ailments like tuberculosis, sexually transmitted diseases, fever, diabetes, inflammation, and malaria (Luseba and Merwe, 2006; Mogale et al., 2019; Papo et al., 2022; Semenya and Maroyi, 2012). The Bapedi and Tsonga people in Limpopo use the roots of *Z. zeyheriana* as a remedy for diarrhea, internal parasites, tuberculosis, and other respiratory infections (Semenya and Maroyi, 2019). The stem, root, and leaf decoctions of *Z. rivularis* are traditionally used for the treatment of dysentery, respiratory diseases, swollen glands, wounds, sores, and snake bites in parts of Venda and Mpumalanga (Constant and Tshisikhawe, 2018; Hutchings and Scott, 1996; Palmer and Pitman, 1972).

Different studies have demonstrated the *in vitro* activity of *Z. mucronata* against bacteria, fungi, and parasites, including *Plasmodium* (Abolaji et al., 2021; Buthelezi et al., 2024). Major phytoconstituent classes identified in *Z. mucronata* include flavonoids, peptide alkaloids, and triterpenoids (Auvin et al., 1996; Fehlhaber et al., 1972; Ibrahim and Islam, 2017). To our knowledge, the antiplasmodial activity and cytotoxicity of *Z. rivularis* and *Z. zeyheriana* have not been studied. Hence, this study assessed these *Ziziphus* species' antiplasmodial activity and cytotoxicity. Furthermore, ^{1}H NMR-based metabolomics and GC-MS analyses were employed to identify phytoconstituents that can be correlated with the observed bioactivity.

2. Materials and methods

2.1. Plant sample collection

Ziziphus mucronata was collected from the Vhembe District Municipality in Limpopo Province (22°00′ S, 29°12′ E). *Ziziphus rivularis* and *Z. zeyheriana* were sourced from the Manie Van Der Schijff Botanical Garden (25°45′ S, 28°13′ E) and the University of Pretoria Experimental Farm (25°00′ S, 28°00′ E), respectively. Ms. Magda Nel performed the taxonomic authentication of all collected species, and voucher specimens of *Z. mucronata* (130883), *Z. rivularis* (130882), and *Z. zeyheriana* (0125140) were deposited in the HGWJ Schweickerdt Herbarium (PRU). Samples of root bark, stem bark, and leaves were collected from each plant species, except for *Z. zeyheriana*. When *Z. zeyheriana* was collected, its foliage had withered; thus, only the root bark and stem bark were obtained. Eight plant samples from the three species were collected, air-dried, and finely ground into powder using an Ultracentrifugal Mill (Retsch, Germany) and stored in brown paper bags.

2.2. Plant sample extraction

Each plant sample (40 g) underwent sequential extraction using five different solvents in the following sequence: (1) *n*-hexane, (2) dichloromethane, (3) ethyl acetate, (4) ethyl acetate: methanol (1:1; v/v), and (5) methanol (Maria et al., 2018). For each extraction, 400 ml of solvent was added to the weighed plant material, homogenized for 10 min using a blender (Philips, Netherlands), then sonicated in an ultrasonic water bath (Labotec, Midrand) for an additional 10 min, and finally filtered through Whatman No.1 filter paper (Merck, Germany). Each sample was subjected to three extraction cycles before proceeding to the next solvent. The solvent in the resulting filtrate was evaporated using a rotary evaporator (Buchi, R-200, Switzerland), and the residue was transferred to glass vials. Additionally, separate decoctions were prepared for each plant sample to mimic traditional preparation methods and enable comparative analysis with the organic extracts (Zhang et al., 2018). Approximately 40 g of plant material was boiled at 100 °C for 45 min, cooled, and filtered into a glass jar. The resulting decoction samples were frozen overnight at −80 °C before drying in a manifold freeze dryer (Virtis, New Zealand). All obtained decoction extracts were stored in glass vials at room temperature. Forty-eight extracts were obtained from sequential and traditional decoction extraction methods.

2.3. [^{3}H]hypoxanthine incorporation assay

All 48 successive extracts were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 1 mg/ml and subsequently subjected to [^{3}H]hypoxanthine incorporation assay to assess their *in vitro* antiplasmodial activity against *P. falciparum* NF54, with chloroquine (Sigma-Aldrich C6628) as a positive control (Desjardins et al., 1979; Ponnudurai et al., 1981). The samples were cultured in RPMI 1640 medium devoid of hypoxanthine. The RPMI 1640 medium (5.95 g/L), neomycin (100 U/ml), $NaHCO_3$ (2.1 g/L), and human erythrocytes (0.3 % parasitemia) were supplemented with HEPES. Eleven 3-fold serial dilutions of the plant extracts, ranging from 50 to 0.001 μg/ml, were prepared in 96-well microtiter plates before incubating in a humidified atmosphere with 4 % CO_2, 3 % O_2, and 93 % N_2 at 37 °C. This humidified environment maintained a suitable temperature of 37 °C. After an hour, 50 μl of hypoxanthine was added to each well, followed by further incubation for 24 h. The erythrocytes were harvested using a BetaplateTM harvester (Wallac, Australia), rinsed with distilled water on a glass fiber filter, and then placed on a plastic foil containing dry glass filters with 10 ml of scintillation fluid for cell counting. The IC_{50} values were determined using linear regression from sigmoidal curves with the aid of Softmax Pro Software (Huber and Koella, 1993).

2.4. Cytotoxicity screening

Rat skeletal myoblast cell lines were used to evaluate for cytotoxicity of all 48 extracts, with podophyllotoxin (Sigma P4405) used as a positive control. In microtiter plates, approximately 100 μl of RPMI 1640 medium supplemented with 1 % L-glutamine (200 nM), 10 % fetal bovine serum, and 4000 rat skeletal myoblast cells were seeded (Ahmed et al.,

1994). Eleven three-fold serial dilutions of the plant extracts were prepared, covering concentrations ranging from 100 to 0.002 μg/ml, and the microtiter plates were incubated for 70 h. Following incubation, the plates were examined under an inverted microscope to evaluate normal cell growth. About 10 μg/ml of resazurin was added after the plates were incubated for 2 h. The plates were analyzed after incubation using the Spectramax Gemini XS microplate fluorometer (Molecular Devices, California) with excitation and emission wavelengths of 536 nm and 588 nm, respectively. The CC_{50} values were determined using linear regression from sigmoidal curves with the aid of Softmax Pro Software (Huber and Koella, 1993).

2.5. ^{1}H NMR spectroscopy and multivariate data analyses

All 48 successive extracts were analyzed using ^{1}H NMR spectroscopy to compare the chemical profiles depicted by the different chemical shifts. Each extract was then re-suspended in deuterated dimethyl sulfoxide (DMSO-d_6) to a final concentration of 15 mg/ml. The individual spectra were recorded on a 400 MHz spectrometer (Varian, California) at a temperature of 25 °C. The spectral width was set to 14 ppm, and 64 scans were acquired for each sample. Manual magnetic shimming was performed to ensure consistent spectral resolution across all spectra (Heyman et al., 2015). MestReNova (Version 14.20, Mestre lab) software was employed to process the obtained spectra for Multivariate Data Analysis (MDA). This processing involved manual phase correction, baseline correction using the Whittaker smoother method, and referencing all spectra to the DMSO-d_6 (2.5 ppm) solvent peak (Eilers, 2003). Signal regions corresponding to the solvent (2.400–2.600 ppm) and the water peak (3.200–3.400 ppm) were excluded from the analysis. Each spectrum was segmented into bins of 0.04 ppm and then exported to Soft Independent Modeling (SIMCA-P, version 4.1) software (Umetrics, Sweden) for generating Principal Component Analysis (PCA), Orthogonal Partial Least Squares – Discriminant Analysis (OPLS-DA), and contribution plots.

2.6. GC-MS analysis

Two extracts from each plant, displaying the best antiplasmodial activity ($IC_{50} \leq 5$ μg/ml), were subjected to GC-MS analysis to identify specific phytoconstituents attributed to the observed activity between *Z. mucronata*, *Z. rivularis*, *and Z. zeyheriana*. These were resuspended in the respective analytical grade solvents. The GC-MS analysis instrument used was the GCMS-QP2010SE, which used an AOC-20i/s autosampler (Shimadzu, Japan). The splitless injection technique delivered 1 mg/ml of each sampled extract into the GC-MS instrument. Constituents were identified based on the relative mass spectral data comparison with those of the National Institute of Standards and Technology (NIST) database (NIST, 2023).

3. Results

The 50 % inhibitory concentrations (IC_{50}) of the plant extracts against the chloroquine-sensitive *P. falciparum* NF54 strain, L6 rat skeletal myoblast cells, and the selectivity indices (SI) are summarized in Table 1.

3.1. In vitro antiplasmodial activity

Five of the 48 tested successive extracts displayed high antiplasmodial activity ($IC_{50} \leq 5$ μg/ml), while 12 and 17 extracts exhibited promising (5 μg/ml < $IC_{50} \leq 20$ μg/ml) and moderate (20 μg/ml < $IC_{50} \leq 50$ μg/ml) activity, respectively (Table 1). The remaining extracts were inactive against the *P. falciparum* NF54 strain ($IC_{50} > 50$ μg/ml). All tested decoctions displayed no significant antiplasmodial activity compared to the organic extracts, except for *Z. zeyheriana* root bark extract, which displayed promising activity at IC_{50} of 12.6 μg/ml.

Table 1
The inhibitory concentration of 50 % of South African *Ziziphus* species against *P. falciparum* NF54 (IC_{50}), mammalian L-6 cells (CC_{50}), and their selective indices (SI). The indicated IC_{50} and CC_{50} values are the means of two independent assays (n = 2) conducted in triplicate.

Plant (Part)	Sample ID (Solvent)	IC_{50} (μg/ml)[a]	CC_{50} (μg/ml)[b]	SI
Z. mucronata (Leaf)	ZML1 (*n*-hexane)	22.7 ± 1.1	>100	ND
	ZML2 (Dichloromethane)	11.0 ± 2.6	58.6 ± 2.4	5
	ZML3 (Ethyl acetate)	12.1 ± 0.4	69.3 ± 0.7	6
	ZML4 (Ethyl acetate: Methanol (1:1))	>50	>100	ND
	ZML5 (Methanol)	>50	63.9 ± 4.1	ND
	ZML6 (H_2O)[c]	>50	80.1 ± 1.1	ND
Z. mucronata (Stem bark)	ZMS1 (*n*-hexane)	5.78 ± 3.1	22.3 ± 0.9	4
	ZMS2 (Dichloromethane)	3.04 ± 0.1	16.2 ± 0.1	5
	ZMS3 (Ethyl acetate)	4.47 ± 0.1	19.8 ± 2.5	4
	ZMS4 (Ethyl acetate: Methanol (1:1))	>50	54.1 ± 3.7	ND
	ZMS5 (Methanol)	43.2 ± 6.9	51.3 ± 2.1	1
	ZMS6 (H_2O)[c]	>50	66.4 ± 17.9	ND
Z. mucronata (Root bark)	ZMR1 (*n*-hexane)	8.54 ± 0.3	54.8 ± 6.6	6
	ZMR2 (Dichloromethane)	3.43 ± 0.5	24.0 ± 4.1	7
	ZMR3 (Ethyl acetate)	10.5 ± 1.2	69.7 ± 17.6	7
	ZMR4 (Ethyl acetate: Methanol (1:1))	33.8 ± 0.4	49.5 ± 3.5	2
	ZMR5 (Methanol)	36.2 ± 7.6	49.8 ± 5.1	1
	ZMR6 (H_2O)[c]	>50	52.8 ± 0.1	ND
Z. rivularis (Leaf)	ZRL1 (*n*-hexane)	>50	>100	ND
	ZRL2 (Dichloromethane)	>50	>100	ND
	ZRL3 (Ethyl acetate)	>50	>100	ND
	ZRL4 (Ethyl acetate: Methanol (1:1))	7.48 ± 2.1	49.5 ± 1.8	7
	ZRL5 (Methanol)	11.3 ± 1.4	50.1 ± 2.4	4
	ZRL6 (H_2O)[c]	27.1 ± 1.9	46.1 ± 3.1	2
Z. rivularis (Stem bark)	ZRS1 (*n*-hexane)	32.2 ± 9.9	>100	ND
	ZRS2 (Dichloromethane)	21.1 ± 8.3	>100	ND
	ZRS3 (Ethyl acetate)	42.6 ± 0.2	61.6 ± 11.3	3
	ZRS4 (Ethyl acetate: Methanol (1:1))	25.3 ± 3.5	72.6 ± 21.8	3
	ZRS5 (Methanol)	>50	53.3 ± 7.1	ND
	ZRS6 (H_2O)[c]	>50	>100	ND
Z. rivularis (Root bark)	ZRR1 (*n*-hexane)	41.1 ± 1.3	>100	ND
	ZRR2 (Dichloromethane)	27.8 ± 0.4	>100	ND
	ZRR3 (Ethyl acetate)	36.7 ± 2.3	>100	ND
	ZRR4 (Ethyl acetate: Methanol (1:1))	34.5 ± 6.4	51.7 ± 2.5	2
	ZRR5 (Methanol)	>50	68.2 ± 26.8	ND
	ZRR6 (H_2O)[c]	>50	>100	ND
Z. zeyheriana (Stem bark)	ZZS1 (*n*-hexane)	9.7 ± 0.8	56.7 ± 10.4	6

(*continued on next page*)

Table 1 (*continued*)

Plant (Part)	Sample ID (Solvent)	IC_{50} (μg/ml)[a]	CC_{50} (μg/ml)[b]	SI
	ZZS2 (Dichloromethane)	5.89 ± 0.1	58.1 ± 1.3	10
	ZZS3 (Ethyl acetate)	5.76 ± 1.1	67.0 ± 8.3	12
	ZZS4 (Ethyl acetate: Methanol (1:1))	25.7 ± 9.8	47.2 ± 0.4	2
	ZZS5 (Methanol)	23.5 ± 0.7	61.7 ± 17.4	3
	ZZS6 (H_2O)[c]	12.6 ± 0.1	>100	ND
Z. zeyheriana (Root bark)	ZZR1 (*n*-hexane)	10.3 ± 0.3	55.1 ± 6.2	5
	ZZR2 (Dichloromethane)	4.94 ± 0.1	19.1 ± 0.1	4
	ZZR3 (Ethyl acetate)	3.25 ± 0.1	15.7 ± 0.4	5
	ZZR4 (Ethyl acetate: Methanol (1:1))	22.7 ± 1.9	48.4 ± 0.9	2
	ZZR5 (Methanol)	35.0 ± 3.3	65.6 ± 0.2	2
	ZZR6 (H_2O)[c]	>50	46.1 ± 0.5	ND
Chloroquine[d]		0.002		
Podophyllotoxin[e]			0.008	

ND –Not Determined.
[a] *P. falciparum* (NF54) strain.
[b] Rat skeletal myoblast human cell lines (L-6 cell lines).
[c] Decoction.
[d] Antiplasmodial assay positive control.
[e] Cytotoxicity assay positive control.

Overall, the ethyl acetate stem bark extract of *Z. mucronata* had the highest antiplasmodial activity at 3.04 μg/ml. This was followed by the ethyl acetate root bark extract of *Z. zeyheriana* with IC_{50} of 3.25 μg/ml. *Ziziphus rivularis* showed no significant activity, but the ethyl acetate: methanol (1:1; v/v) and methanol extracts displayed promising activity at IC_{50} of 7.48 and 11.3 μg/ml, respectively (Table 1). In *Z. mucronata* and *Z. zeyheriana*, bioactivity was mostly observed in the non-polar extracts of the different plant parts. In *Z. rivularis*, bioactivity was only observed in the polar leaf extracts. These findings show that moderate antiplasmodial activity is common among *Ziziphus* species in South Africa.

3.2. *In vitro cytotoxicity screening*

There was no apparent cytotoxicity in 43 of the 48 tested extracts at $CC_{50} > 50$ μg/ml (Table 1). The remaining extracts exhibited $CC_{50} < 50$ μg/ml, indicating cytotoxicity against L6 rat skeletal cells. Although a poor selectivity (SI < 10) trend was prevalent, the DCM and EtOAc stem bark extracts of *Z. zeyheriana* displayed selectivity with SI of 10 and 12, respectively (Table 1). These findings suggest that *Z. mucronata, Z. rivularis, and Z. zeyheriana* species have no apparent cytotoxicity, at least *in vitro*. However, to further ascertain their clinical safety, *in vivo* studies will need to be conducted.

3.3. ^{1}*H NMR-based metabolomics analysis*

Metabolomics based on ^{1}H NMR spectral data was conducted only for the root and stem extracts. This was done to minimize data skewing; hence, the leaves were excluded because they displayed no significant antiplasmodial activity. The root and stem bark extracts were prioritized because traditional people commonly use them for malaria treatment, while the leaves are less frequently utilized (Mokgolodi et al., 2011). For the analysis, samples with $IC_{50} > 20$ μg/ml were also excluded to increase the model's robustness and fitness.

The unsupervised PCA model displayed no discrimination between active and non-active samples in the training set (Fig. 1). The model displayed R^2 and Q^2 values of 0.65 and 0.37, respectively. Suggesting that the data fits perfectly in the model ($R^2 \geq 0.5$), however, the model is not dependable in predicting antiplasmodial activity ($Q^2 < 0.5$). Thus, a supervised OPLS-DA was generated. Discrimination between active and non-active samples in the training set was observed after the OPLS-DA algorithm was applied, with R^2 and Q^2 values of 0.8 and 0.7, respectively (Fig. 2). The R^2 and Q^2 values obtained in the OPLS-DA model affirm its robustness and fitness.

A contribution plot was then generated from the OPLS-DA data to identify the different classes of compounds attributed to the observed antiplasmodial activity (Table 1). Phytoconstituent classes attributed to the observed antiplasmodial activity include aliphatic (0–1.5 ppm), allylic (1.6–1.9 ppm), methyl ketone (2–2.2 ppm), and carboxylic acid (11–12 ppm) based phytoconstituents (Fig. 3). The aliphatic-based constituents are more abundant, followed by the carboxylic acid-based compounds, indicating that antiplasmodial activity is mostly attributed to constituents of these classes.

3.4. *GC-MS analysis of the most active extracts*

Gas chromatography-mass spectrometry was conducted to identify antiplasmodial phytoconstituents. Not all extracts were tested. However, two of the best activities were selected from each plant. The chromatograms of the analyzed extracts, indicating the retention times (Rt) of the different constituents, are shown in Figs. 4–6. In *Z. mucronata*, antiplasmodial activity is attributed to lupeol and palmitic acid. Lupeol (Rt = 12.9 min) and palmitic acid (Rt = 19.0 min) were both determined in extract ZMS2 (Fig. 4A). In extract ZMS3, only lupeol (Rt = 25.6 min) is attributed to the observed antiplasmodial activity (Fig. 4B).

For *Z. rivularis*, bioactivity is associated with lupeol and friedelin. Lupeol was observed in extracts ZRL2 and ZRL4 at a retention time of 26.1 min. Friedelin was identified in ZRL4 at a retention time of 31.0 min (Fig. 5B).

In *Z. zeyheriana*, activity is attributed to palmitic acid, identified in both ZZR2 and ZZR3 extracts. Palmitic acid was eluted from ZZR2 and ZZR3 at a retention time of 20.4 min (Fig. 6A and B).

Lupeol and friedelin are terpenoids, which are aliphatic-based compounds, while palmitic acid is a long-chain fatty acid found abundantly in most plant species. These results suggest that lupeol, friedelin, and palmitic acid significantly contribute to the observed antiplasmodial activity, correlating with the bioactive classes previously identified in Fig. 3.

4. Discussion

Malaria is treatable, yet the burden continues to increase in Africa, especially in the sub-Saharan region (Li et al., 2024). Despite the availability of various antimalarials, the eradication of malaria continues to prove difficult to achieve. Widespread antimalarial drug resistance further complicates the malaria burden (Nguyen et al., 2023). Thus, the need to constantly discover novel and effective antimalarial agents is imperative (Umumararungu et al., 2023). Medicinal plants have long been a source of nutrition and healing for humanity. Today, modern pharmaceuticals rely on these medicinal plants to supply antimalarial bioactive ingredients (Habibi et al., 2022; Tajbakhsh et al., 2021).

The current study investigated the antiplasmodial activity and cytotoxicity of *Ziziphus mucronata, Z. rivularis,* and *Z. zeyheriana*, which have a documented history of ethnomedicinal use among the Swazi, Zulu, Tsonga, Bapedi, and Venda communities in South Africa. These species are used to treat various ailments, including malaria. The findings corroborate the ethnomedicinal use of *Z. mucronata*, aligning with previous published research. For example, Zininga et al. (2017) investigated the stem bark of *Z. mucronata* and reported activity on the hexane and 50 % ethyl acetate (1:1) at an IC_{50} of 13.9 μg/ml against

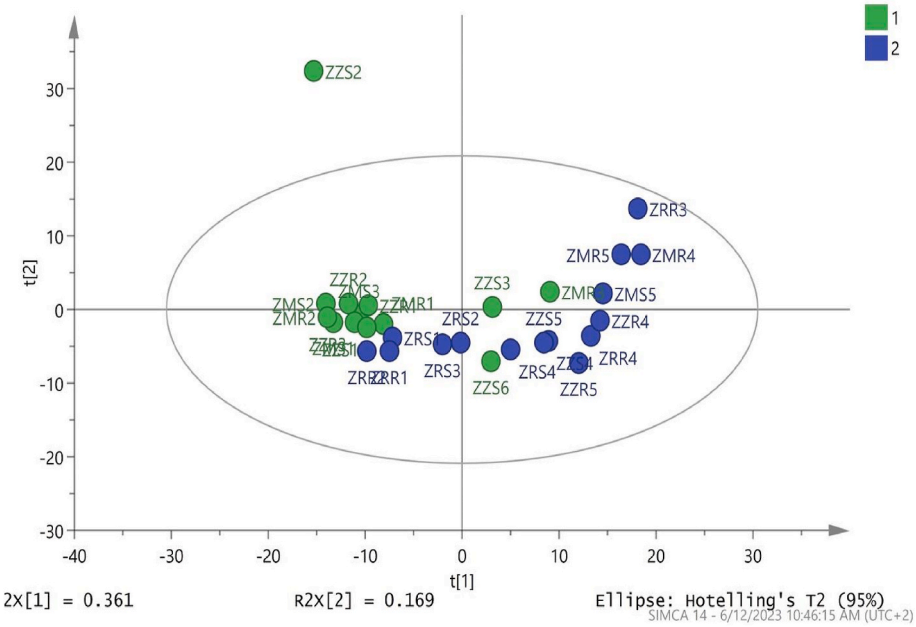

Fig. 1. The PCA scores plot of selected extracts *Z. mucronata, Z. rivularis,* and *Z. zeyheriana* extracts: (1) – active and (2) – non-active. (Observations: N = 28, Variables: K = 352 (X = 350, Y = 2)).

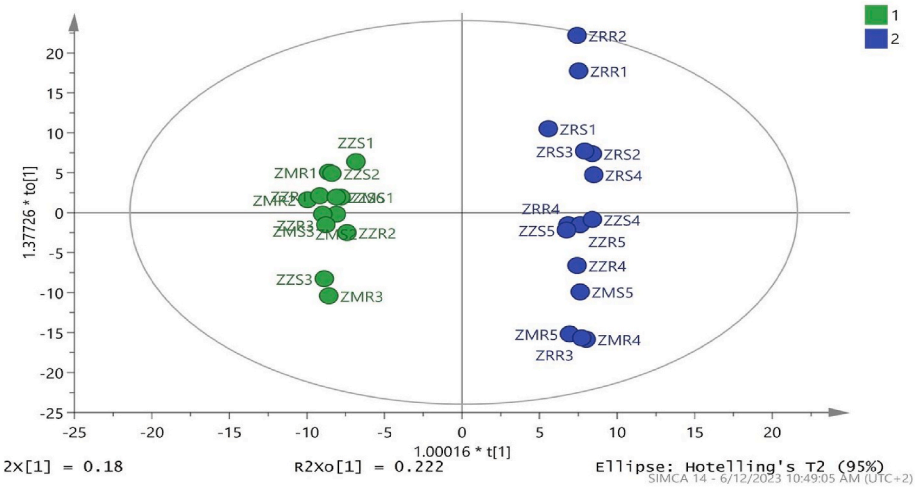

Fig. 2. OPLS-DA plot showing the discrimination between the active and non-active extracts from *Z. mucronata, Z. rivularis* and *Z. zeyheriana*: (1) – active and (2) – non-active. (Observations: *N* = 28, Variables: K = 352 (X = 350, Y = 2)).

P. falciparum 3D7. Similarly, Clarkson et al. (2004) assessed the DCM leaf extract of *Z. mucronata* against the D10 strain of *P. falciparum* and reported antiplasmodial activity at 12 μg/ml, which further correlated with the findings of this study. Although the tests were not conducted against *P. falciparum* NF54, the findings affirm the antiplasmodial potential of *Z. mucronata*. Although *Z. rivularis* and *Z. zeyheriana* have not been previously evaluated, the findings suggest that moderate antiplasmodial activity is shared commonly among these *Ziziphus* species. However, it is unclear whether the observed activity is due to synergistic interactions or if there are active constituents that may exhibit significant activity when isolated.

Despite these *Ziziphus* species exhibiting no apparent cytotoxicity, they display poor selectivity (SI < 10). The selectivity index (SI) is a ratio of the toxic concentration of the extracts against the bioactive concentration (Dzoyem et al., 2016). The higher the SI, the more acceptable an extract is as an antiplasmodial lead. However, poor parasite selectivity, as with our extracts, does not necessarily mean the extracts may not be further pursued for antiplasmodial activity (Radha and Jawad, 2021). It has been shown in some studies that extract derivatization, downstream fractionation, and pure compound isolation from such extracts may enhance bioactivity, reduce cytotoxicity, and increase selectivity (Kou et al., 2018; Said et al., 2024). This could be the case with our samples, as has been shown in the downstream fractionation of *Z. mucronata* previously (Zininga et al., 2017).

The R^2 and Q^2 values obtained from the PCA and OPLS-DA plots represent two significant components. The R^2 value measures how well the data fits in the model, and Q^2 measures how well the model predicts antiplasmodial activity (Eriksson et al., 2013). Ideally, in biological samples, $R^2 \gg Q^2$, and R^2 should not be greater than Q^2 by a factor of more than 0.2 (Worley and Powers, 2013). In this PCA plot (Fig. 1), the R^2 and Q^2 values of 0.65 and 0.37, respectively, demonstrate the model's fitness and inability to predict antiplasmodial bioactivity. Regardless of the data fitting perfectly, the poor prediction capabilities lessen the PCA model's ability to predict antiplasmodial bioactivity. The

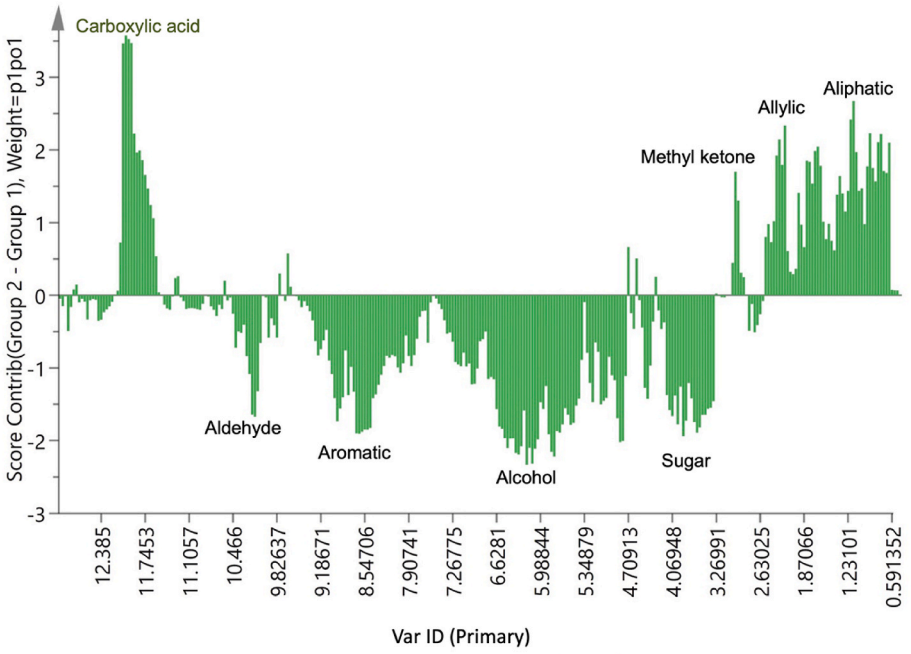

Fig. 3. Contribution plot showing the major classes of compounds attributed to the observed antiplasmodial bioactivity of *Z. mucronata, Z. rivularis,* and *Z. zeyheriana*. (Active – bars projecting upwards and non-active – bars projecting downwards).

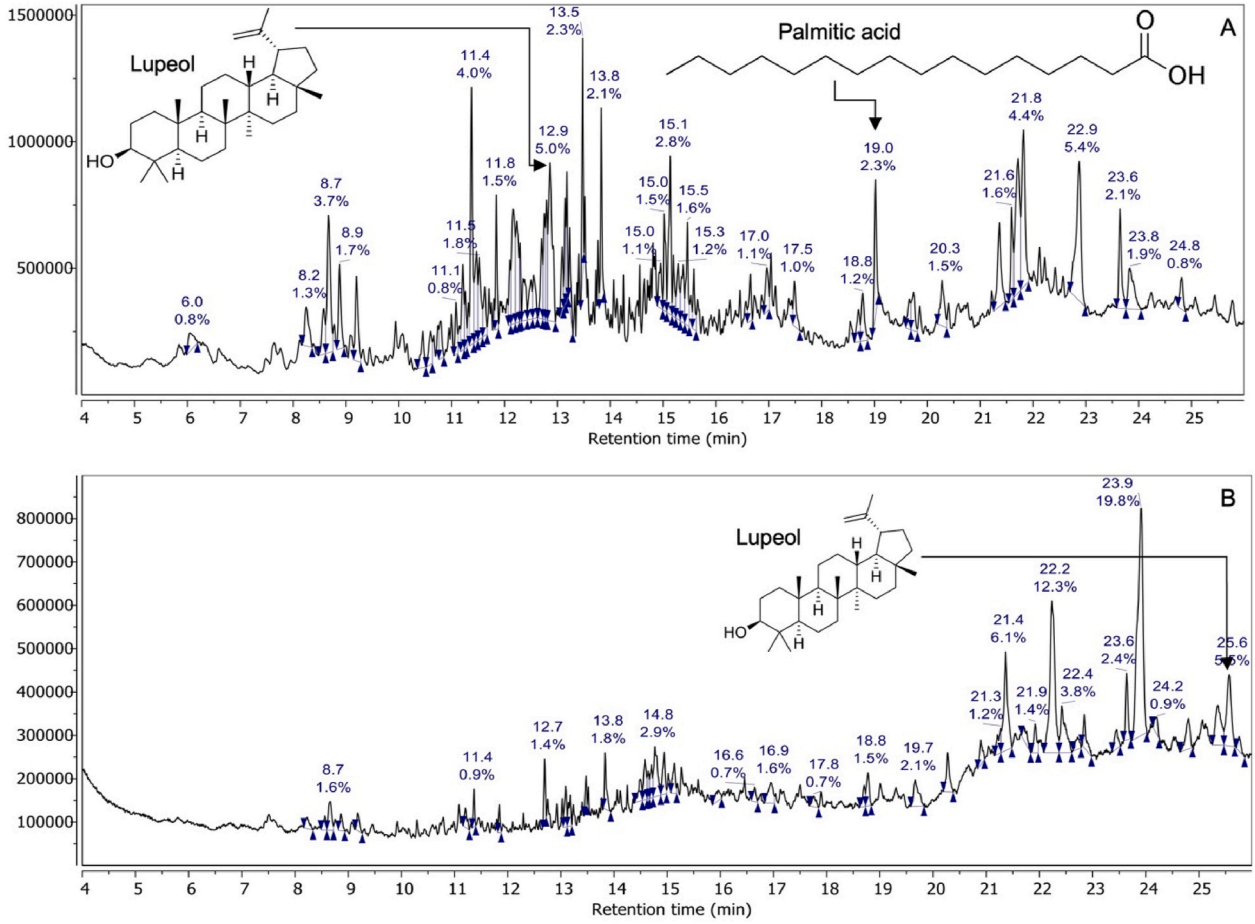

Fig. 4. Chromatograms showing the identified antiplasmodial constituents in *Z. mucronata* stem bark extracts: (A) extracted using dichloromethane and (B) extracted using ethyl acetate.

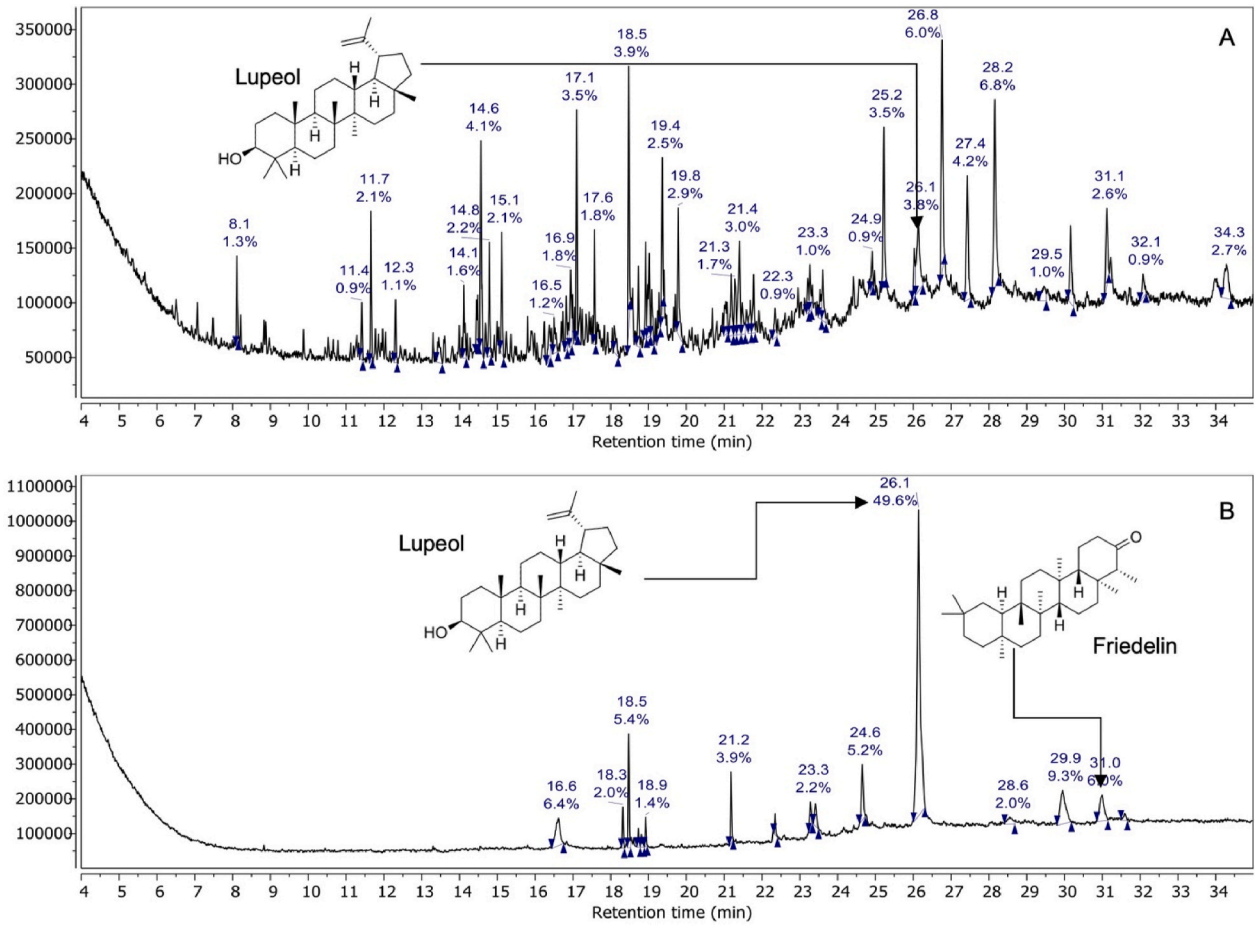

Fig. 5. Chromatograms displaying the identified antiplasmodial compounds in *Z. rivularis* leaf extracts: (A) Extracted using dichloromethane and (B) extracted using ethyl acetate: methanol (1:1).

OPLS-DA approach discriminated better between the active and non-active samples in the training set (Fig. 2). This was evident in the R^2 and Q^2 values of 0.8 and 0.7, respectively, affirming the OPLS-DA model's ability to discriminate according to the observed antiplasmodial activity (Table 1). Relying on the R^2 and Q^2 values alone to determine the model's reliability is not sufficient (Worley and Powers, 2016). Further cross-validation of the OPLS-DA model was conducted using CV-ANOVA, employing the Student's T-test. The obtained *P*-value of 0.05 was within the acceptable range, further confirming the model's reliability in predicting antiplasmodial activity.

From the OPLS-DA model, the contribution plot tentatively identified aliphatic, carboxylic acid, allylic, and methyl ketone-based classes of compounds. This was further correlated by the GC-MS analysis, which attributed the observed antiplasmodial activity to lupeol (aliphatic/terpenoid), palmitic acid (fatty acid), and friedelin (aliphatic/terpenoid). Lupeol, palmitic acid, and friedelin are known for their established antiplasmodial activity. Lupeol was isolated from the leaves of *Ficus benjamina* and displayed an IC_{50} of 3.8 μg/ml against the *P. falciparum* 3D7 strain (Singh et al., 2020). Palmitic acid from *Vernonia amygdalina* was assessed *in vivo* against *P. berghei ANKA*. A good chemo-suppression of 71.58 % was observed at the lowest dose of 10 mg/kg for palmitic acid administered orally (Afolayan et al., 2024). However, palmitic acid has a dose-dependent cytotoxicity; that is, at higher doses, particularly 100 mg/kg, it results in notable inflammatory and structural alterations in the kidneys and liver as well as elevated genotoxic markers in bone marrow cells (Afolayan et al., 2024). Similar to the effects of cyclophosphamide, the 100 mg/kg dose resulted in severe kidney damage, including glomerular and tubular disruptions, and marked increases in micronucleated polychromatic erythrocytes, whereas low doses caused mild hepatic and renal alterations. According to these results, palmitic acid is comparatively safe at lower dosages but may be harmful to the body at higher doses (Afolayan et al., 2024). That being considered, palmitic acid as a potential candidate can still be pursued through chemical derivatization methods to reduce its cytotoxicity. Friedelin was evaluated against *P. falciparum* K1 and W2 strains and displayed IC_{50} values of 7.7 and 7.2 μM, respectively (Ngouamegne et al., 2008). Lupeol and friedelin also displayed moderate interactions with some P450 cytochrome metabolizing enzymes when subjected to *in silico* molecular docking studies, further affirming their drug-like properties (Baah et al., 2024). The occurrence of these phytoconstituents in *Z. mucronata*, *Z. rivularis*, and *Z. zeyheriana* affirms the observed antiplasmodial activity in these species. These findings are significant in that they have unearthed the antiplasmodial potential of *Z. rivularis* and *Z. zeyheriana*, which were previously not studied.

5. Conclusion

The study has demonstrated antiplasmodial activity and poor cytotoxicity of *Z. mucronata*, *Z. rivularis*, and *Z. zeyheriana*. Integrating ^1H NMR metabolomics and GC-MS analyses made the identification of active constituent classes and pure compounds possible. The observed bioactivity is partly attributed to lupeol, palmitic acid, and friedelin constituents, previously recognized for their antiplasmodial activity. This is the first study to report antiplasmodial activity in *Z. rivularis* and *Z. zeyheriana*. Further, chromatographic fractionation and isolation from *Z. rivularis* and *Z. Zeyheriana* are underway to target unexplored antiplasmodial phytoconstituents. These will be subjected to *in vitro* and *in vivo* antiplasmodial screening and cytotoxicity assessment.

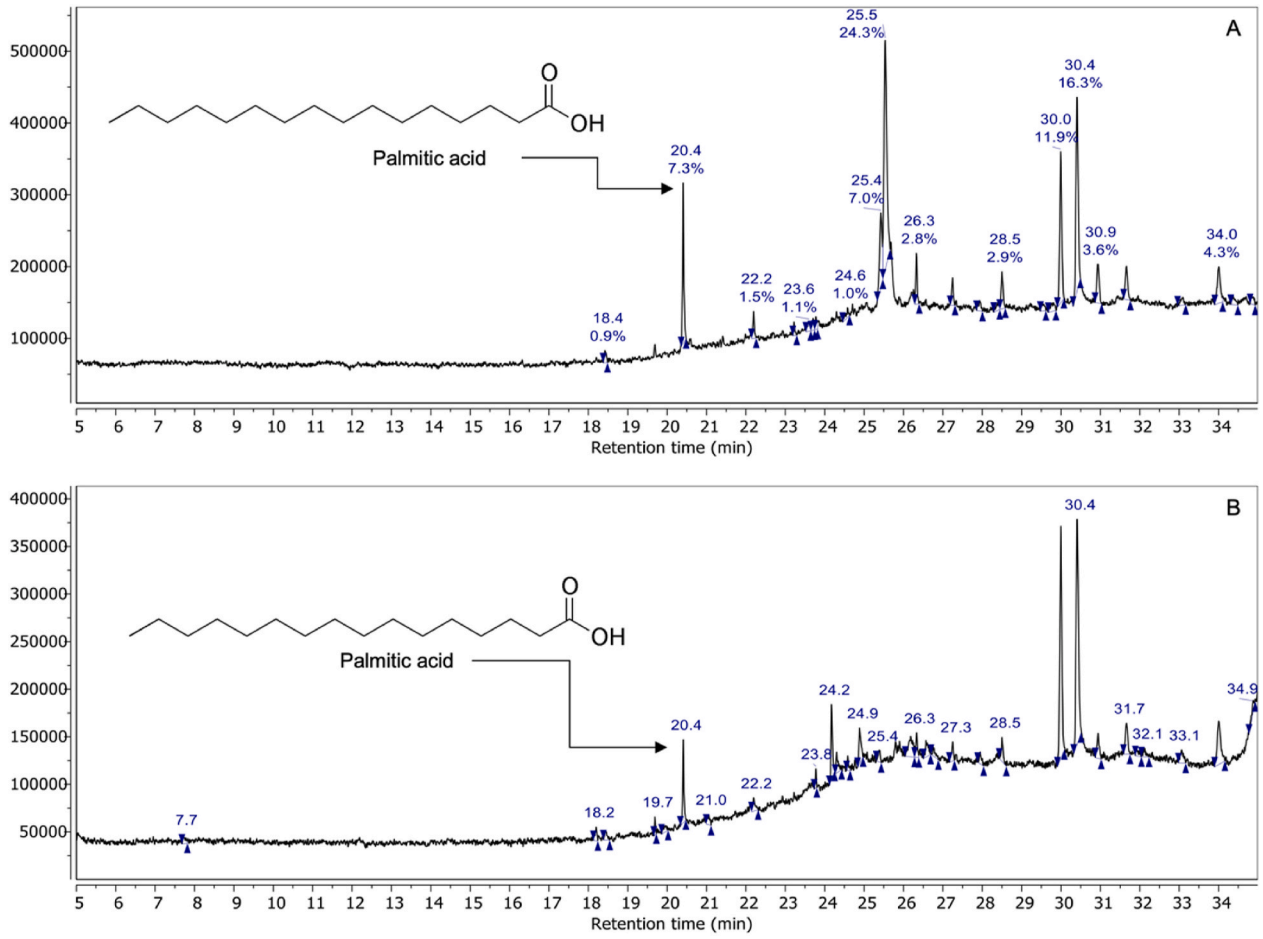

Fig. 6. Chromatograms displaying the identified antiplasmodial compounds in *Z. zeyheriana* root extracts: (A) extracted using dichloromethane and (B) extracted using EtOAc.

CRediT authorship contribution statement

M.J. Mabuza: Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Writing – original draft. **M. Kaiser:** Methodology. **M.J. Bapela:** Supervision, Resources, Writing – review and editing, Funding acquisition, Conceptualization. **T.E. Tshikalange:** Writing – review and editing, Funding acquisition, Resources, Acquisition. **A.A. Yusuf:** Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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