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EVALUATION OF ANTIOXIDANT AND IN VITRO ANTI-INFLAMMATORY ACTIVITIES OF HEXALOBUS MONOPETALUS (A. RICH.) ENGL. & DIELS EXTRACTS

SÉVERIN MBAÏHOUGADOBÉ[1,2], ABEL MBAÏOGAOU[2], MIHAELA DRAGOI CUDALBEANU[3,4], BÉTOLOUM SALOMON MADJITOLOUM[2], DANIELA BORDA[5], ANDREEA VERONICA BOTEZATU[3,*], BIANCA FURDUI[3,*], YAYA MAHMOUT [2], RODICA MIHAELA DINICĂ[3]

[1]*Laboratory of Life Chemistry (LaC&V) University of Moundou, Tchad, BP 206 Moundou, Chad*

[2]*Research Laboratory of Natural Substances, FSEA of University of N'Djaména, Chad. BP: 1117 N'DJaména Chad*

[3]*Department of Chemistry, Physics and Environment, Faculty of Sciences and Environment, "Dunarea de Jos" University of Galati, 111 Domnească Street, 800201 Galati, Romania*

[4]*Faculty of Land reclamation and Environmental Engineering, University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, 011464, Bucharest, Romania*

[5]*Faculty of Food Science and Engineering, "Dunărea de Jos University of Galati", 111 Domnească Street, 800201, Galati, Romania*

[*]Corresponding authors*:* bfurdui@ugal.ro, andreea.botezatu@ugal.ro

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Abstract

Hexalobus monopetalus is a medicinal plant which grows in tropical Africa, whose chemical composition and biological properties are not yet fully known. It is traditionally used for alleviating gastric disorders, fevers and diabetes. Phytochemicals responsible for the biological activities are found in varying levels in many medicinal plants. This study aimed to evaluate various extracts from the roots of *H. monopetalus* from Chad, central Africa. Phenolic compounds were extracted by two methods, one being hydromethanolic (H_2O/MeOH) extraction and the second method an extraction with four solvents of increasing polarity: cyclohexane, dichloromethane, ethyl acetate, and methanol. The antioxidant capacity was determined by DPPH and ABTS methods. Total polyphenols were assessed by the Folin-Ciocalteu method, and also, the anti-inflammatory activity was estimated *in vitro*. Gas chromatography coupled with mass spectrometry (GC-MS) analysis showed that the cyclohexane extract contains a predominance of polycyclic sesquiterpenes, and other compounds such as alcohols and phenols, thus demonstrating the richness and chemical diversity of the extract. Polyphenol content was higher in the dichloromethane extract (523.53 mg EqQ/g). Total flavonoid content in the water-methanol extract (20:80 v/v) was 227.84 mg EqQ/g dry matter, 183.85 mg EqQ/g dry matter was found in the methanolic extract, and in the ethyl acetate extract there was 178.93 mg EqQ/g dry matter. The antioxidant and anti-inflammatory properties

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also varied depending on the extraction solvent. The hydromethanolic extract was the most active in all cases, while the dichloromethane extract showed less antioxidant activity.

Keywords: *Hexalobus monopetalus*, antioxidants, anti-inflammatory activity, Chad medicinal plants

Introduction

Since the dawn of time, humans have appreciated the soothing and analgesic properties of plants. For Africa in general, and Chad in particular, traditional medicine represents a cultural and economic heritage of undeniable importance. These considerations are still valid in Chad, where almost 75-80% of the population lives in rural areas where conventional healthcare is scarce. In this geographical area, patients generally travel long distances to reach a medical facility due to limited resources, including the high cost of vaccines, diagnostics, medical devices, and routine pharmaceuticals. These challenges have led to an increased use of herbal medicines by patients in Africa (Tome, 2015; Kasilo *et al.*, 2019).

Hexalobus monopetalus (HM) is a small tree, mainly found in tropical Africa, 8 to 10 m high, with black, longitudinally fissured bark, known as “Baboons' breakfast” or “Shakama plum” (Dzoyem *et al.*, 2016). The plant is spread in Senegal, Cameroon, South Africa, Nigeria, and Southeast Mozambique. In Chad, the plant is found in the southwest, quite frequently in Sudanian zones (César and Chatelain, 2019), and it is widely used in traditional medicine (Bonnet and Arbonnier, 2008; Arbonnier, 2009; Mireku *et al.*, 2016; Mbaïhougadobe, 2017; Nguemo Dongock *et al.*, 2018).

Indole alkaloids known as hexalobines have been isolated from the roots and fruits of *H. monopetalus* (Achenbach *et al.*, 1995; Malebo *et al.*, 2014; Mireku *et al.*, 2016). Tests carried out on compounds isolated from the root barks of this plant have demonstrated antimalarial effects against *Plasmodium falciparum* K1 strains (multidrug-resistant) and NF54 strains (sensitive to chloroquine), with IC_{50} values of 9.9 and 13 µg/mL respectively, antifungal and trypanocidal activity against *Trypanosoma brucei rhodesiense*, with a minimum inhibitory concentration (MIC) of 11 µg/mL (Malebo *et al.*, 2014).

The *in vitro* study of anti-inflammatory and antioxidant activities occupies a crucial place in research to identify potential new molecules to combat inflammatory diseases and oxidative damage. Inflammation and oxidation are two closely linked biological mechanisms, which play an essential role in human health and pathology.

This study aimed to determine the chemical composition, anti-inflammatory and antioxidant activities of root bark crude and fractional extracts of *Hexalobus monopetalus* obtained in solvents of different polarity.

Materials and methods

Plant material

Hexalobus monopetalus root barks were harvested in March 2019 from the South of Moundou town, between Mankou and Faya, two villages in the Logone Oriental

region in Boro canton, from Chad, Africa. They were cut into small pieces, dried at room temperature, ground until a fine powder was obtained, and stored in airtight jars for later use.

Preparation of extracts

For both extraction methods, 20 g of dried plant powder were subjected to extraction in 200 mL of solvent and stirred for twenty-four hours, then filtered. A crude hydromethanolic (H_2O/MeOH) extract was prepared under those conditions by using the water/methanol (20/80) system. The fractional extraction with solvents of increasing polarity was performed by using the plant material residue left after each extraction and performing the extraction with the next solvent. The solvents used were cyclohexane, dichloromethane, ethyl acetate, and methanol. The extracts were concentrated using a rotary evaporator for obtaining cyclohexane extract, dichloromethane extract (HM DCM), ethyl acetate extract (HM AcOEt), and methanol extract (HM MeOH), respectively. Thus, five different extracts were obtained, namely a crude extract (hydromethanolic) and four fractions. The extracts were concentrated and stored at 4°C for subsequent analyses.

Phytochemical study

Phytochemical screening was carried out by High Performance Thin Layer Chromatography (HPTLC) (Wagner and Bladt, 1996; Bălănescu *et al*., 2022) and gas chromatography-mass spectrometry (GC-MS).

HPTLC analysis

Semiautomated CAMAG HPTLC application device equipped with HPTLC scanner winCATS software version 1.4.9, and Linomat V autosampler (CAMAG, Muttenz, Switzerland) was used to determine a qualitative screening of the extracts obtained. Aluminium plates pre-coated with silica gel 60 F254 (E. MERCK KGaA) measuring 20.0 x 10.0 cm were used. The optimized mobile phase was a mixture of toluene: ethyl acetate: formic acid: methanol (3: 4 : 0.8 : 0.7). This mobile phase was selected among others from literature for the better separation of the compounds in the analyzed extracts. Samples were applied to the plates using the CAMAG Linomat 5 applicator and a 100 µL CAMAG micro-syringe. The spots were applied at 1.5 cm from each other and 2 cm from the bottom edge. The CAMAG ADC2 "ADC2 Development" chromatographic chamber was pre-saturated with the mobile phase for 2.0 minutes. The development distance on a TLC plate was 80 mm and the elution time was 5 minutes. Spectrometric analysis was performed over a wavelength range from 200 to 800 nm. The scanning speed was 100 nm/s. Quercetin, gallic acid, chlorogenic acid, and malvidin were used as standard pure commercial compounds (Sigma Aldrich, Darmstadt, Germany).

Chemical analysis by GC-MS

Cyclohexane root barks extracts of *H. monopetalus*, which give off a strong odor and have an oily appearance, were analyzed by GC-MS with the aim of determining their chromatographic profile and volatile compound composition (Qin *et al.*, 2012).

Aliquots of 10 µL sample were transferred to headspace vials (15 mL) and 10 µL of 2-octanol (Sigma Aldrich Chemie GmbH, Steinheim, Germany) was added as

internal standard, along with 2.50 g of saturated $(NH_4)_2SO_4$. Each glass vial was sealed with a spectrum seal and an aluminum cap. Solid Phase Micro Extraction (SPME) was performed using a carboxene-polydimethylsiloxane (CAR/ PDMS, 75 µm) fiber from Supelco (Bellefonte, PA, USA). The fiber was conditioned according to the manufacturer's instructions and thermally cleaned for 50 minutes at 250°C in the conditioning station of the post-pass autosampler.

Volatile compounds were extracted after incubation of the vials for 30 minutes at 50°C, followed by exposure of the SPME fiber to the sample headspace at 20 mm depth in the vial and at 50°C for 20 minutes. For thermal desorption, the SPME fiber was inserted into the ingestion port of the GC. A desorption time of 4 minutes at 250°C was observed in splitless mode. Volatile compounds (VOCs) in cyclohexane extracts of HM were analyzed using a Trace GC-MS Ultra ITQ 900 gas chromatograph from Thermo Scientific (USA) coupled to a selective mass (MS) detector with ion trap (Thermo). The GC column was a TG-WAX capillary column (60 m × 0.25 mm, i.d. 0.25 µm). Helium (99.996% purity, Messer S.A., Romania) was used as carrier gas at a flow rate of 1 ml/min. The furnace temperature was programmed as follows: isothermal treatment at 40°C for 4 min, followed by an increase to 50°C at 3°C/min and 120°C at 5°C/min, then to 170°C at 7°C/min and finally to 220°C at 10°C/min, with the temperature held constant for 8 minutes. The furnace was then cooled to the initial temperature. The temperature of the MS transfer line was set at 270°C. Mass spectra were obtained from the full scan of positive ions resulting from an electron impact (EI) of 200 eV with a scan range of 50 to 650 m/z. The GC-MS system is controlled by Xcalibur software. The compounds obtained were tentatively identified by comparing the mass spectra with those in the Wiley and Nist 08 library database, with an acceptable similarity rate.

Determination of Total Phenolic and Total Flavonoid Contents

The assays were carried out using a 96-well plate reader (Tecan Pro M200, Tecan Trading AG, Männedorf, Switzerland). The instrument was controlled by Tecan i-control software.

Total polyphenols determination was performed using the Folin-Ciocalteu method (Agbor *et al.*, 2014; Zongo *et al.*, 2023). First, 25 µL of Folin-Ciocalteu reagent was added to 10 µL of each extract. After 5 minutes of incubation, 25 µL of a 20% aqueous sodium carbonate solution and 140 µL of distilled water were added up to a total volume of 200 µL. The blank consisted of 10 µL of the extract, 25 µL of 20% sodium carbonate and 165 µL of distilled water. Sample absorbance values at 760 nm were recorded after 30 minutes of incubation. Freshly prepared gallic acid solutions of different concentrations (500 - 0.97 µg/mL) were used to plot the calibration curve. Results were expressed in milligram equivalents of gallic acid per gram of dry matter (mg GAEq/g DW).

Flavonoid quantification was determined using $AlCl_3$ assay (Cudalbeanu *et al.*, 2018; Silihe *et al.*, 2022). Briefly, 100 µL of aqueous $AlCl_3$ solution (2%) was added to 100 µL of extracts or standard solution. Absorbance was measured after 15 min incubation at 415 nm against a blank consisting of 100 µL water and 100 µL aqueous $AlCl_3$ (2%) solution. Flavonoid concentrations were determined from the quercetin

calibration curve (40 - 0.078 µg/mL). Results are expressed as milligram quercetin equivalent per gram dry matter (mg EqQ/g DW).

In vitro evaluation of biological activities

Determination of antioxidant activity

Antioxidant activity was assessed *in vitro* using electron/hydrogen transfer methods. These are the DPPH (2,2`-diphenyl-1-picrylhydrazyl) free radical scavenging, and the ABTS (2,2'-azinobis-(3-ethyl-BenzoThiazoline-6-Sulfonic Acid) methods.

DPPH radical scavenging method is based on free radical inhibition ($DPPH^{\bullet}$), measured by the reduction in absorbance at 517 nm linked to paired electron resonance (Gulcin, 2020). In a 96-well plate, 100 µL of 300 µM DPPH solution (in 95% methanol) were added to 100 µL of extracts at different concentrations. The resulting solutions were then homogenized and left in the dark at room temperature. Absorbance values were recorded at 517 nm after 20 min, 60 min and 120 min of incubation. The control consisted of a 1:1 mixture of methanol and DPPH solution. The blank sample consisted of a mixture of 1:1 methanol and extracts for each sample. A lower absorbance indicated greater free radical scavenging activity (Dinică *et al.*, 2021; Kedare and Singh, 2011).

Percentage inhibition was determined using formula 1:

$$\text{DPPH inhibition (\%)} = \left[1 - \left(\frac{DOsample}{DOcontrol}\right)\right] \times 100 \quad (1)$$

Antiradical activity was also assessed by the $ABTS^{\bullet+}$ radical cation decolorization assay (Crespo *et al.*, 2019; Busuioc *et al.*, 2023). ABTS was dissolved in distilled water at a concentration of 7 nM. The $ABTS^{\bullet+}$ radical cation solution was obtained by incubating for 12-16 h in the dark at room temperature an equal volume mixture of the ABTS stock solution with a 2.45 nM potassium persulfate solution. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.700 ± 0.02 at 734 nm before use. Then, 1.5 mL of $ABTS^{\bullet+}$ solution was mixed with 50 µL of extract or reference (gallic acid) at different concentrations (250, 125, 62.5, 31.25, 15.62, 7.81 µg/mL). Absorbances were measured at 734 nm after a 10-minute incubation in the dark at room temperature. Three assays were performed for each concentration of tested products, and the results were expressed as a percentage of inhibition (I%) as previously described for the DPPH assay.

In vitro anti-inflammatory activity: egg albumin denaturation method

The plant's anti-inflammatory activity was assessed *in vitro* by the egg albumin denaturation test induced by heat treatment. The reaction mixture consisted of 8 µL albumin, 112 µL PBS (pH 6.3), and 80 µL extract 50 mg/mL. Distilled water was used instead of extract as a negative control. The mixtures were incubated at 37°C for 15 minutes, then heated to 70°C for 5 minutes. After cooling, absorbance was measured at 660 nm (Osman *et al*., 2016). Two measurements were carried out after cooling at 30 min and then at 120 min on Tecan Pro M200 against a blank not containing the extracts.

The percentage inhibition of protein denaturation was calculated as follows:

$$\% \text{ Denaturation inhibition} = \left(1 - \frac{DOsample}{DOcontrol}\right) \times 100 \quad (2)$$

where DOsample: absorbance of test sample; DOcontrol: absorbance of negative control.

Results and discussion

Phytochemical study

Phytochemical screening by HPTLC

The qualitative identification of phytoconstituents by HPTLC analysis allows the visualization of individual components present in a sample. The HPTLC chromatograms show quenching at 254 nm, indicating the presence of conjugated double bonds in nonaromatic or aromatic compounds. The blue, yellow, and red colors could explain the presence of phenolic compounds such as coumarins, flavonoids, anthraquinones, and others. The red spots observed at $R_f > 0.5$ could explain the presence of triterpenes and anthocyanins in the dichloromethane extract (Wagner *et al*., 1996). The high absorption at around R_f 0.80 in dichloromethane extract could explain the presence of polyphenols and flavonoids in higher concentrations. The increased concentration in these compounds observed in the fractioned extracts could be attributed to the superior solubility of phytochemical compounds in various solvents compared to the hydromethanol system.

Figure 1 shows the chromatograms obtained at 254 nm (green background) and at 365 nm (blue background) as well as the corresponding densitograms of these HPTLC chromatograms (Figures 1a, 1b, 1c).

HPTLC was used to detect the phytochemical components in the extracts of *H. monopetalus*. These results show the separation of 11 compounds for DCM extract, 10 compounds for the AcOEt extract and 13 compounds for the hydromethanol extract, demonstrating the presence of different classes of interest such as polyphenols, terpenoids or flavonoids. Figure 1 shows the presence of quercetin in all samples, with a spot with Rf value of 0.75 ± 0.04, in DCM extract (11 in figure 1.a), in AcOEt extract (8 in figure 1.b) and in hydromethanolic extract (12 in figure 1.c). Gallic acid was identified at a Rf value of 0.64 ± 0.05 in DCM extract (10 in figure 1.a) and in hydromethanolic extract (10 in figure 1.c). The presence of chlorogenic acid was shown with a spot with Rf value of 0.2 ± 0.08 in DCM extract (4 in figure 1.a), in AcOEt extract (4 in figure 1.b) and in hydromethanolic extract (4 in figure 1.c). Malvidin was identified at Rf value of 0.28 ± 0.05 in DCM extract (5 in figure 1.a), in AcOEt extract (5 in figure 1.b) and in hydromethanolic extract (5 in figure 1.c). This analysis demonstrated the presence of quercetin, gallic acid, chlorogenic acid, and malvidin in the studied extracts. In addition, the TLC chromatograms showed the presence of other spots of unidentified compounds in the analyzed extracts indicating the variation in phytochemical constituents between different *H. monopetalus* extracts, thus requiring further quantitative analysis.

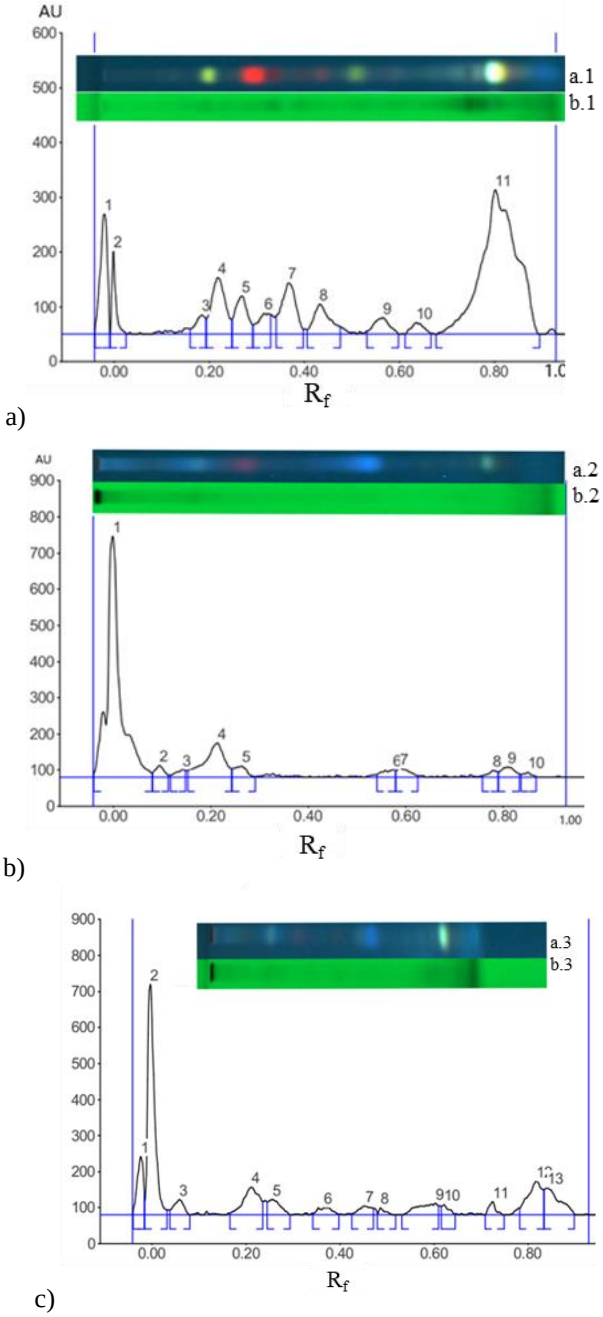


Figure 1. Chromatograms under UV visualization at 365 nm (a.1, b.1, c.1) and 254 nm (a.2, b.2, c.2), and densitograms obtained for DCM (a), AcOEt (b) and H_2O/MeOH (c) extracts. The numbers from each peak represent the number of spots corresponding to the number of compounds that were separated from each extract, respectively 11 (a), 10 (b), and 13 (c) spots.

Polyphenols and flavonoids are secondary plant metabolites that are widely found in various plant parts and are also basic components of the human diet. Many phytochemicals isolated from various natural sources are considered to have therapeutic potential for many diseases, with quercetin being one of the most popular and biologically active of them. Quercetin is a widespread and extensively studied flavonoid found in various food sources, including fruits, vegetables, seeds, and nuts (Wang *et al.*, 2022). Anthocyanidins are flavonoids widely present in fruits and vegetables. Cyanidin, delphinidin, malvidin, peonidin, petunidin, and pelargonidin are the six common anthocyanidins that are potentially useful as nutraceutical ingredients because they provide numerous beneficial health effects (Mattioli *et al.*, 2020). These classes of secondary plant metabolites have demonstrated diverse biological properties, including antioxidant, anti-inflammatory, antibacterial, antiviral, radical scavenging, gastroprotective, and immunomodulatory activities, which are of interest to the pharmaceutical, cosmetic and food industries (Wang *et al.*, 2022; Mattioli *et al.*, 2020). The presence of these classes of compounds was demonstrated in this study, to our knowledge, for the first time in extracts from the bark roots of *H. monopetalus*, which may justify the use of this plant in traditional medicine and may encourage further studies of this plant species.

Chemical analysis of cyclohexane extracts of Hexalobus monopetalus (A. Rich.) Engl. & Diels by GC-MS

The analysis of the GC-MS profile of the cyclohexane extract of *H. monopetalus* root bark shows that this extract contains a wide variety of chemical compounds, including alcohols, aldehydes, arenes (such as ethylbenzene), monoterpenes, sesquiterpenes, phenols, esters, and sterols (Figure 2).

A total number of 59 compounds were identified using NIST library and representing ~ 99% of the total volatiles present in the mixture from which 28 terpenes, sesquiterpenes and derivatives presented in Table 1.

The most intense peak (RT 36.31 min) corresponds to a polycyclic sesquiterpene (dehydro-aromadendrene). Its mass spectrum (Figure 3) shows a base peak at m/z 159 with a relative abundance of 100%. This compound has a molecular peak at m/z 202.

Phytochemical analysis by GC-MS shows that the major identified compounds are: 8,9-dehydro-cycloisolongifolene (RT=36.31 min, Peak area 30.84%) which is a polycyclic sesquiterpene representing almost a third of the chemical composition of the extract, polycyclic sesquiterpenes 8-cedren-13-ol (RT=36.7 min, Peak area 7.11%), and bergamotol (RT=36.7 min, Peak area 7.11%), followed by 1,2,3,4,4a,5,6,8a-octahydro-naphthalene (RT=42.88 min, Peak area 8.29%). Most identified compounds belong to the sesquiterpene class (α-patchoulene with RT 31.4 min) and polycyclic sesquiterpenes (calarene, aromandendren, cadinene, γ-murolene, ledene). The peaks between 37.85 and 41.92 min correspond, on the same bases, to compounds of the nuciferol type, an aromatic alcohol, and sterols. This is typical of essential oils and aromatic plant extracts (Clarke, 2008; Toumi *et al.*, 2011). Other families of compounds were identified, notably aromatic alcohols, phenolic compounds, polyunsaturated esters, and heterocycles. Dehydro-

aromadendrene compound from the intense peak (RT = 36.31 min) is also found in the *Eucalyptus* family, mainly in *Eucalyptus globulus* (Clarke, 2008).

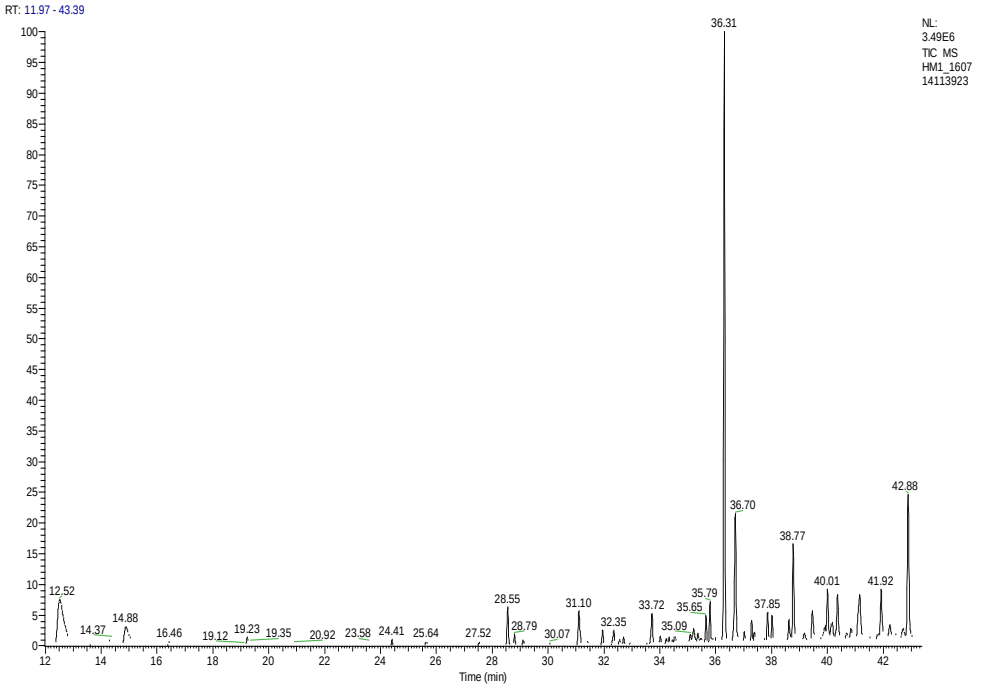


Figure 2. Chromatographic profile of the cyclohexane extract of *H. monopetalus* root bark

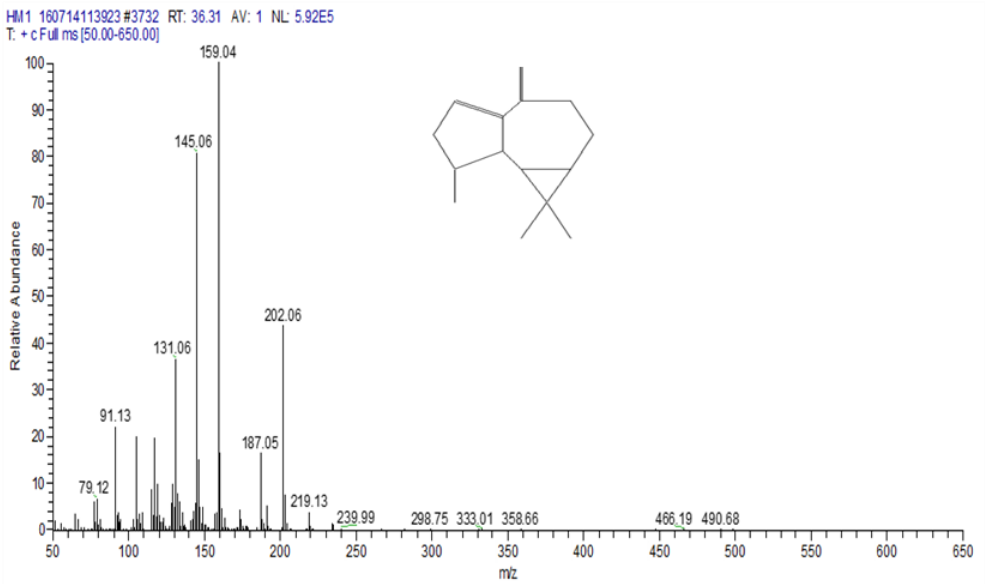


Figure 3. Mass spectrum and structure of the dehydro-aromadendrene compound from the intense peak (RT = 36.31 min)

Table 1. Tentative chemical composition of cyclohexane extracts analyzed by GC-MS

RT (min)	Peak area %	Formula	Name	Class of compounds
12.52	2.91	$C_8H_{18}O$	2-ethyl-4-methyl-1-pentanol	alcohols
14.36	0.48	$C_8H_{14}O$	4,4-dimethyl-2-methylene-valeraldehyde	aldehydes
16.47	0.33	$C1_0H_{16}$	2,5-dimethyl-3-methylene-1,5-heptadiene	acyclic monoterpenes
18.83	0.08	C_8H_{10}	Ethylbenzene	arenes
20.92	0.13	C_8H_{10}	o-xylene	arenes
22.8	0.06	$C_{10}H_{16}$	1-methyl-4-(1-methylethyl)- 1,4-cyclohexadiene	cyclic monoterpenes
23.02	0.04	$C_{10}H_{16}$	7-Propylidene-bicyclo[4.1.0]heptane	monoterpenes
23.58	0.24	$C_{11}H_{20}O$	2-methoxy-1,7,7-trimethyl-bicyclo[2.2.1]heptane	monoterpenoids
24.19	0.05	C_9H_{12}	1-ethyl-2-methyl-benzene	arenes
25.49	0.04	$C_{10}H_{14}$	1,3,8-p-menthatriene	cyclic monoterpenes
25.64	0.25	$C_{12}H_{18}O_2$	Myrtenyl acetate	monoterpenic esters
26.81	0.08	$C_{10}H_{14}$	1,3,8-Menthatriene	cyclic monoterpenes
28.56	2.13	$C_{12}H_{22}$	1-heptyl- Cyclopentene	cycloalchenes
29.1	0.31	$C_{15}H_{24}$	alpha-Cubebene	sesquiterpenes
30.07	0.16	$C_{15}H_{24}$	Copaene	sesquiterpenes
31.1	1.98	$C_{15}H_{24}$	1,2,4a,5,8,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-,(1à,4aà,8aà)-(n)- Naphtalene	bicyclic sesquiterpenes
31.76	0.05	$C_{15}H_{24}O$	8-Cedren-13-ol	bicyclic alcohol sesquiterpenes
31.95	0.77	$C_{11}H_{16}O$	2-methoxy-4-methyl-1-(1-methylethyl)-benzene	Phenolic ether (2-isopropyl-4-methyl-anisole)
32.56	0.28	$C_{11}H_{16}O$	2-methoxy-4-methyl-1-(1-methylethyl)-benzene	Phenolique ether (Anisole, 2-isopropyl-4-methyl-)
32.7	0.36	$C_{15}H_{24}$	1,3a-Ethano-3aH-idene,1,2,3,6,7,7a-hexahydro-2,2,4,7a-tetramethyl-,[1R-(1à,3aà,7aà)]-	sesquiterpenes (α-patchoulene)
32.96	0.18	$C_{15}H_{24}$	[1aR-(1aà,4à,4aà,7bà)]-1a,2,3,4,4a,5,6,7b-octahydro-1,1,4,7-tetramethyl-1-H-cycloprop[e]azulene	polycyclic sesquiterpenes (Ledene)
33.09	0.06	$C_{15}H_{24}$	α-Guaiene	bicyclic sesquiterpene
33.5	0.16	$C1_5H_{24}$	α -Guaiene	bicyclic sesquiterpene
33.72	1.82	$C_{15}H_{22}O$	6-(p-Tolyl)-2-methyl-2-heptenol	(Nuciferol) aromatic alcohol
34.01	0.42	$C_{15}H_{24}$	[3aR-(3aà, 4à,7à)]-2,4,5,6,7,8-hexahydro-1,4,9,9-tetramethyl-3H,3a,7-methanoazulene	bicyclic sesquiterpenes (Cedrene)
34.32	0.36	$C_{15}H_{24}$	(-)-Aristolene	polycyclic sesquiterpenes
35.21	0.80	$C_{15}H_{24}$	(-)-Aristolene	polycyclic sesquiterpenes
35.36	0.46	$C_{15}H_{24}$	Guaiene	bicyclic sesquiterpenes

RT (min)	Peak area %	Formula	Name	Class of compounds
35.65	1.28	$C_{15}H_{24}$	(1S-cis)-1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-Napthalene	bicyclic sesquiterpenes (Cadinene)
35.79	1.98	$C_{15}H_{24}$	Isoledene	polycyclic sesquiterpenes
36.31	30.84	$C_{15}H_{22}$	8,9-dehydro-Cycloisolongifolene	polycyclic sesquiterpenes
36.7	7.11	$C_{15}H_{24}O$	8-Cedren-13-ol	polycyclic sesquiterpenes
36.7	7.11	$C_{15}H_{24}O$	Bergamotol	polycyclic sesquiterpenes
37.28	1.00	$C_{12}H_{18}O_2$	2-(1,1-dimethylethyl)-1,4-dimethoxy- benzene	diphenolique ether
37.6	0.10	$C_{22}H_{32}O_2$	Doconexent	fatty acide w3 (Docosahexaenoic acide, cervonic acid)
37.71	0.10	$C_{24}H_{32}O_5$	(3à, 5à, 11à, 15à)-14,15(epoxy-3,11-dihydroxy-bufa-20,22-dienolide,	Stérol compound (Marinobufagenin)
37.85	1.49	$C_{15}H_{22}O$	6-(p-tolyl)-2-methyl-2-heptenol	(Nuciferol) aromatique alcool
38.01	1.19	$C_{17}H_{26}O_2$	2,6-Bis(1,1-dimethyl)-4-(1-oxopropyl)phenol	phenol (antioxydante)
38.32	0.13	$C_{20}H_{22}O_6$	Columbin	diterpenoide (bitter)
38.62	1.12	$C_{19}H_{30}O_2$	Methyl-6,8-octadecadiynoate	polyunsaturated ester
38.78	5.22	$C_{15}H_{24}O$	8-Cedren-13-ol	Sesquiterpene bicyclique alcool
39.17	0.36	$C_{24}H_{40}O_2$	methylester -10,12-Tricosadiynoic acide	Polyunsaturated ester
39.46	1.65	$C_{15}H_{24}O$	8-Cedren-13-ol	bicyclic sesquiterpene alcohol
40.01	2.58	$C_{15}H_{22}O$	2,2,7,7-Tetramethyltricyclo [6.2.1.0(1,6)]unde-4-en-3-one	Polycyclic sesquiterpeniques cetone
40.16	0.91	$C_{26}H_{40}O_2$	Butyl-4,7,10,13,16,19-docosahexaenoate	Ester
40.36	2.32	$C_{15}H_{22}O$	6-(p-Tolyl)-2-methyl-2-heptenol	(Nuciferol) aromatic alcohol
40.69	0.28	$C_{23}H_{36}O_2$	i-Propyl 5,8,11,14,17-eicosapentaenoate	polyunsaturated ester
40.85	0.54	$C_{15}H_{24}$	Guaiene	bicyclic sesquiterpene
41.15	2.75	$C_{15}H_{22}O$	6-(p-Tolyl)-2-methyl-2-heptenol	(Nuciferol) aromatic alcohol
41.34	0.25	$C_{21}H_{34}O_2$	(5à)-Androstan-17-one,3-ethyl-3-hydroxy	sterols
41.56	0.11	$C_{21}H_{34}O_2$	(5à)Androstan-17-one,3-ethyl-3-hydroxy	sterols
41.92	2.91	$C_{19}H_{30}O_2$	Methyl 6,8-octadecadiynoate	polyunsaturated ester
42.23	0.82	$C_{15}H_{22}O$	2,2,7,7-Tetramethyltricyclo [6.2.1.0(1,6)]unde-4-en-3-one	sesquiterpenic polycyclic cetones
42.41	0.26	$C_{21}H_{34}O_2$	(5à)-Androstan-17-one,3-ethyl-3-hydroxy	sterols
42.7	0.57	$C_{11}H_{21}N_3O$	N-[6-[N-Aziridyl]-3-aza-3-hexenyl]morpholine	heterocyclic compounds
42.88	8.29	$C_{15}H_{24}$	(1à,4aà,8aà)-Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)	sesquiterpenes
43.39	0.10	$C_{21}H_{34}O_2$	(5à)-Androstan-17-one,3-ethyl-3-hydroxy	sterols

RT (min)	Peak area %	Formula	Name	Class of compounds
44.15	0.36	$C_{17}H_{26}O_2$	2,6-Bis(1,1-dimethyl)-4-(1-oxopropyl)phenol	phenols
44.28	0.04	$C_{21}H_{34}O_2$	(5à)-Androstan-17-one,3-ethyl-3-hydroxy	sterols

GC-MS analysis shows that this extract contains a predominance of polycyclic sesquiterpenes, and other minor compounds such as alcohols and phenols, showing the extract's chemical richness and diversity. The major compounds confer significant pharmacological value to this extract, including anti-inflammatory, antioxidant, antimicrobial, and sedative properties. These effects highlight the therapeutic potential of essential oils rich in oxygenated sesquiterpenes, commonly used in phytotherapy, aromatherapy, and natural dermatology (Zengin *et al.*, 2023).

This information is crucial to understand the potential properties of this extract, whether in terms of its odor, therapeutic effects or applications in various fields such as cosmetics and perfumery.

Quantitative analysis

Determination of total polyphenols

The calibration curve with gallic acid y = 0.003x+0.1924 (R^2 = 0.9982) was used to quantify polyphenols (TPC) in analyzed extracts (DCM, AcOEt, methanol, and hydromethanol). The obtained results are shown in Figure 4. The highest concentration was found in the HM DCM extract, having a higher TPC content than HM hydromethanol extract, HM AcOEt and HM MeOH extracts, respectively.

Determination of total flavonoids

The results of the total flavonoid content (TFC) assay in the various extracts provide information on the quantity of these compounds in the extracts. The levels obtained using the quercetin calibration curve (y = 0.0213x+0.3526, R^2 = 0.993) are shown in Figure 5. These quantitative analysis results indicate that the dichloromethane (DCM) extract is the richest in polyphenols but contains the lowest levels of flavonoids.

The crude hydromethanolic extract was the richest in flavonoids (expressed as quercetin equivalents), while displaying also high levels of polyphenols (expressed as gallic acid equivalents). Ethyl acetate and methanol extracts remained somewhere in between in terms of polyphenol and flavonoid contents. These results confirm those obtained by the qualitative analysis above. The high level of polyphenols in the DCM extract could be explained by a grouping of several families: non-flavonoids (phenolic acids), flavonoids (flavonols, anthocyanins, etc.), and tannins (Iacopini *et al*., 2008).

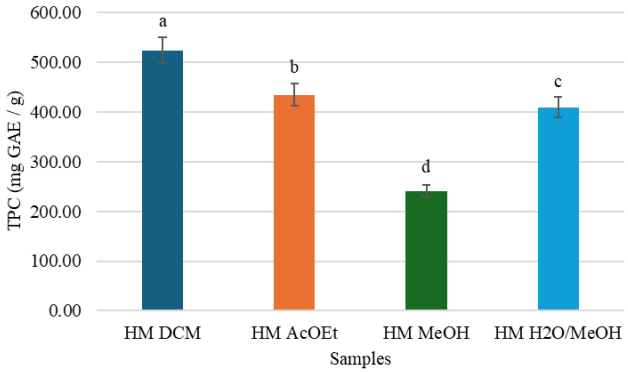


Figure 4. Total polyphenol content in dichloromethane (HM DCM), ethyl acetate (HM AcOET), methanol (HM MeOH), and water/methanol (HM H_2O/MeOH) extracts expressed in mg GAEq/g DW. The values followed by the same letter a, b, c, d show no statistically significant differences ($p < 0.05$) according to the analysis of variance (ANOVA with Tukey's Test). Each value is the mean of three replicates ± standard deviation (SD).

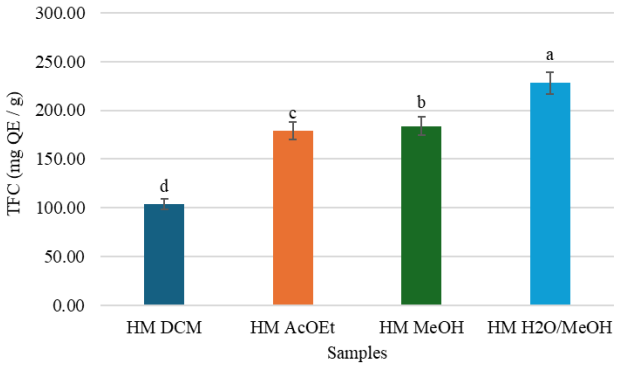


Figure 5. Total flavonoid content in dichloromethane (HM DCM), ethyl acetate (HM AcOET), methanol (HM MeOH), and water/methanol (HM H_2O/MeOH) extracts expressed as mg QE/g DW. The values followed by the same letter a, b, c, d show no statistically significant differences ($p < 0.05$) according to the analysis of variance (ANOVA with Tukey's Test). Each value is the mean of three replicates ± standard deviation (SD).

In vitro evaluation of biological activities

Determination of antioxidant activity

The analysis of the antioxidant activity is essential to assess the ability of compounds to neutralize free radicals and prevent oxidative damage. In this work, two common methods were used to assess antioxidant activity: DPPH and ABTS methods. The results obtained for different extracts at three-time intervals: 20 minutes, 60 minutes, and 120 minutes, using these two methods were examined.

The analysis of the antioxidant activity by the DPPH method is a commonly used technique for assessing the ability of compounds to neutralize free radicals. The

results of DPPH analysis on various extracts as well as on two reference compounds, namely gallic acid and quercetin, are presented in Figure 6.

The results obtained from this test show that the methanolic, ethyl acetate, and hydromethanol extracts have a remarkable antioxidant effect on the DPPH radical from the first 20 minutes. In contrast, the dichloromethane extract showed inhibition levels below 40%. This capacity improved after 2 hours of incubation for this extract.

The results emphasize the significance of incubation time in the analysis of antioxidant properties. Most extracts show an increase in antioxidant activity over time, which can be attributed to a more complete reaction with DPPH radicals. Gallic acid and quercetin stand out for their high antioxidant activity from the outset, suggesting their high capacity to neutralize free radicals. It is also interesting to note that concentration plays a major role in antioxidant activity, and this is particularly for extracts, where higher concentrations are generally associated with better antioxidant activity (Figure 6 and Figure 7).

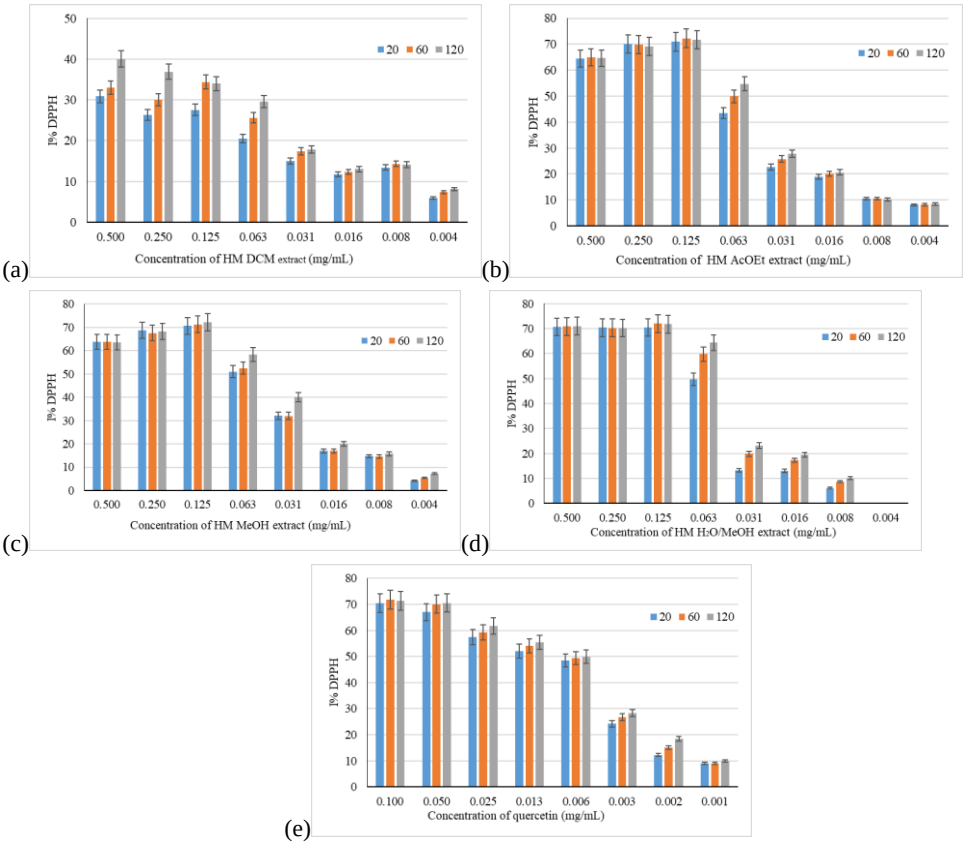


Figure 6. DPPH radical-inhibition activity of root barks extracts from *Hexalobus monopetalus*: a) HM DCM: dichloromethane extract; b) HM AcOEt: extract in ethyl acetate; c) HM MeOH: methanolic extract; d) HM H_2O/MeOH: hydromethanolic extract, and e) quercetin after 20 min, 60 min and 120 min. Each value represents the mean of three replicates ± SD.

The results of the antioxidant activity by the ABTS method of four *Hexalobus monopetalus* extracts and of the gallic acid used as a reference compound are shown in Figure 7. Values are expressed as percent inhibition of antioxidant activity, with gallic acid used as a reference.

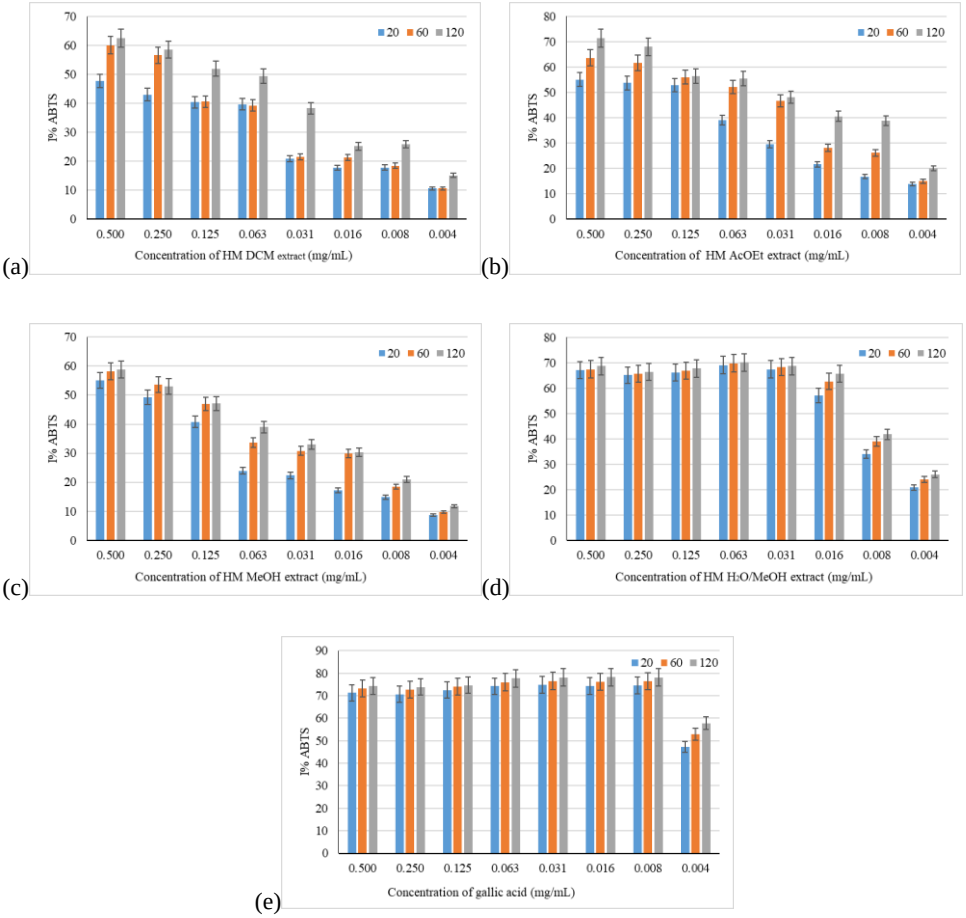


Figure 7. ABTS inhibition activity of root bark extracts from *Hexalobus monopetalus*: a) HM DCM: dichloromethane extract; b) HM AcOEt: extract in ethyl acetate; c) HM MeOH: methanolic extract; d) HM H_2O/MeOH: hydromethanol extract, and e) quercetin after 20 min, 60 min and 120 min. Each value represents the mean of three replicates ± SD.

Over time, the overall results show an increase in the antioxidant activity for most extracts, although some variations are observed. Comparatively, gallic acid has a high antioxidant activity, exceeding that of all extracts tested at most concentrations. The addition of an antioxidant to a solution of this cationic radical results in a reduction of the radical and a decrease in absorbance. This decrease depends on the

antioxidant activity of the compounds tested, time, and concentration (Bibi Sadeer *et al.*, 2020).

Both methods, although complementary, show an increase in antioxidant activity over time, indicating a better reactivity of the extracts with free radicals. It is important to note that the antioxidant performance varies according to the solvent used for extraction in the two methods. For example, hydromethanol extract performs better overall, while dichloromethane extract shows inferior activities. This antioxidant power is most likely due to the high flavonoid content in hydromethanolic extracts, which are known antioxidant substances with the ability to scavenge radical species and reactive forms of oxygen (Heim *et al.*, 2002).

The ABTS radical cation scavenging activity shows similar trends for the antioxidant activity of the extracts, although the results vary. After 20 min, HM hydromethanol and HM AcOEt extracts at a concentration of 0.5 mg/mL showed the highest antioxidant activity values, of 68.621% and 55.074%, respectively.

DPPH and ABTS radical scavenging activities increased in a concentration-dependent manner. The dichloromethane extract showed the lowest activity in both radical scavenging assays. Several phytochemicals, such as polyphenols, flavonoids, sterols, carotenoids, alkaloids, saponins, coumarins, terpenoids, etc., may be responsible for antioxidant activity with health benefits, such as antidiabetic, cardioprotective, anticancer, antiallergic, antimicrobial, and antiviral properties (Kumar *et al*., 2023).

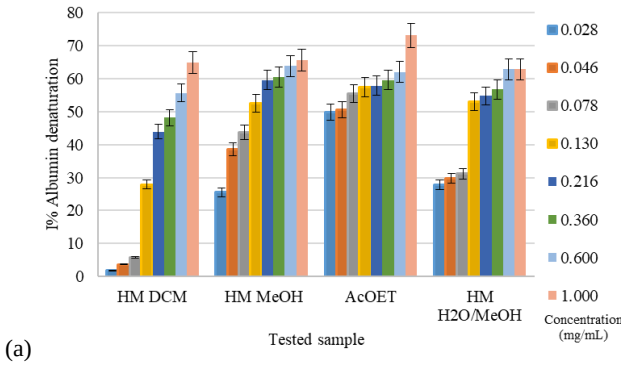
In vitro anti-inflammatory activity: Egg albumin denaturation method

The inhibition percentages of the various extracts (DCM, AcOET, MeOH, H_2O/MeOH) at concentrations ranging from 0.028 to 1.000 mg/mL are shown in Figure 8 after 30 min and 1 h incubation.

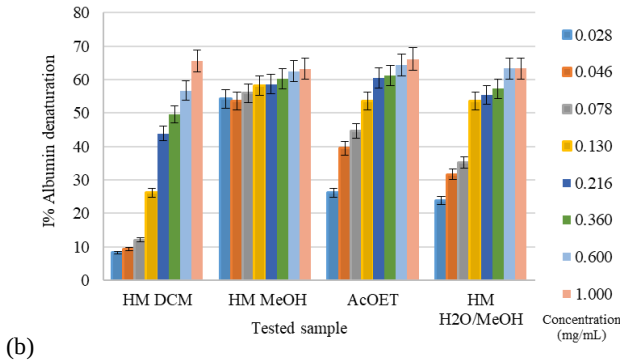
It can be seen in Figure 8 that the albumin-denaturing activity of HM extracts appears to increase with incubation time in all solvents, although the magnitude of this increase may vary depending on the solvent.

The results of the anti-inflammatory method suggest that the effectiveness of these extracts may be dependent on the time and the solvent used for incubation. The study of the anti-inflammatory properties of plants offers immense potential for the discovery of new therapeutic agents. Several studies have focused on identifying the bioactive molecules contained in plant extracts and their ability to modulate inflammatory pathways (Williams *et al.*, 2008). Molecules such as polyphenols (flavonoids), sterols, alkaloids, saponins, coumarins, terpenes, etc., can target enzymes and proteins concentrated in the inflammatory cascade, as well as the production of pro-inflammatory mediators. As well as playing a part in the various inflammatory mechanisms, these substances may also be involved in the production of reactive oxygen species (Abdulkhaleq *et al.*, 2018). This may explain the results obtained from this analysis, where there is a strong inhibition caused by the methanol extract even at very low concentrations. Above 0.5 mg/mL, all extracts inhibited above 50% of the denaturation. The determination of total flavonoids and total polyphenols showed high levels of these compounds in the extracts.

Phytochemical screening and *in vitro* evaluation of the anti-inflammatory and antioxidant activities of HM root barks yielded promising results relevant to exploring the therapeutic potential of this plant in the treatment of various inflammatory and oxidative stress-related diseases.



(a)



(b)

Figure 8. Anti-inflammatory activity, egg albumin denaturation of *H. monopetalus* extracts after 30 min (a) and 1 h (b)incubation. Each value represents the mean of three replicates ±SD.

Studies on this plant species, *Hexalobus monopetalus*, are limited, therefore, the results of this study represent new elements that justify its traditional uses in terms of its chemical composition rich in biologically active compounds.

The various results showed that bark extracts contain polyphenols that could inhibit the production of pro-inflammatory cytokines and reduce the production of pro-inflammatory mediators. Concerning antioxidant activity, the obtained results showed that the extracts possess a strong capacity to trap reactive oxygen species (ROS) and neutralize free radicals. These different actions could be attributed to the presence of compounds such as flavonoids, whose levels are elevated in the hydromethanol extract, phenolic acids, and terpenes, known for their anti-inflammatory properties by targeting inflammatory signaling pathways, and for

being antioxidants capable of protecting cells against oxidative damage (Kumar and Pandey, 2013; Barba-Ostria *et al.*, 2022).

Conclusions

This work led to the characterization of the chemical composition of five different extracts from *Hexalobus monopetalus* roots by using different techniques (UV-Vis, HPTLC, GC-MS), which had not been chemically studied in other studies. Quantitative analysis of extracts in solvents with different polarities showed that *H. monopetalus* bark root had high polyphenol and flavonoid contents. The analysis of antioxidant activity by DPPH and ABTS methods revealed changes in the extracts' capacity to neutralize free radicals. From the evaluation of antioxidant and/or anti-inflammatory capacity, the results show the importance of incubation time and sample concentration. Overall, the hydromethanol extract showed a high level of antioxidant activity that might be due to the high levels of polyphenols present, while the dichloromethane extract showed inferior activities. This variation should be further investigated. These preliminary results provide scientific justification for the traditional use of the plant studied and revealed that *Hexalobus monopetalus* could be a promising source of natural compounds to be further explored for their anti-inflammatory and antioxidant properties.

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