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Phenolics compounds from the roots of *Anthospermum emirnense* Baker (Rubiaceae)

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Abstract

Background: *Anthospermum emirnense* Baker (Rubiaceae), an indigenous and endemic plant of Madagascar, is widely used in traditional Malagasy medicine. The juice extracted from its crushed roots is traditionally applied to treat toothaches and skin infections. The aim of this study was to evaluate its antioxidant activity, quantify its total phenolic content, and isolate and identify its phytochemical constituents.

Methods: The roots of the plant were collected, shade-dried, and pulverized into a fine powder. Sequential extraction was performed by maceration using solvents of increasing polarity: hexane, ethyl acetate, and methanol. Preliminary phytochemical screening of the crude ethanol extract was conducted using standard qualitative methods. The total polyphenol content of the extracts was determined spectrophotometrically using the Folin–Ciocalteu method. Qualitative and quantitative antioxidant tests were performed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. Acute toxicity tests were also carried out.

All experimental results were statistically analyzed using analysis of variance (ANOVA) followed by Tukey's post hoc test. Chromatographic separation of the extracts was performed, and compounds isolated from the ethyl acetate and methanol extracts were identified using nuclear magnetic resonance (NMR) spectroscopy.

Results: Biological assays demonstrated low antioxidant activity for both the ethyl acetate (AcOEt) and methanol extracts, with 50% inhibitory concentrations (IC_{50}) of free radicals measured at 155.02 ± 2.6 µg/mL and 226.53 ± 0.1 µg/mL, respectively. The total phenolic content of each extract was determined to be 168 mg gallic acid equivalents (GAE)/g of dry extract (DE) for the crude ethanol extract, 34 mg GAE/g DE for the ethyl acetate extract, and 38 mg GAE/g DE for the methanol extract. Fractionation of the ethyl acetate and methanol extracts led to the isolation of five known compounds: one anthraquinone, 6-hydroxyrubiadin (1); one naphthoquinone, dehydro-α-lapachone (2); one coumarin, 6-hydroxycoumarin (3); and two iridoids, 8-acetyl-1-epishanzigenin methyl ester (4) and 8-acetylshanzigenin methyl ester (5).

Conclusion: These findings demonstrate the presence of phenolic compounds in the crude ethanolic, ethyl acetate, and methanolic extracts of *Anthospermum emirnense* roots. The extracts exhibit moderate antioxidant activity and show

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signs of acute toxicity. These results highlight the potential of this plant for use in phytomedicine. However, the relatively low antioxidant activity suggests a low polyphenol content.

Keywords: *Anthospermum emirnense*; Phytochemical; Antioxidant; Toxicity

1. Introduction

Anthospermum emirnense Baker (Rubiaceae), commonly known by various local names such as "hazonorana," "hasinorana," "alamiainga," "alaivana," "matoa," "kijanombazimba," or "kisanga," [1]. is a plant traditionally used in Malagasy medicine. The roots of this species are specifically employed in the treatment of common ailments such as toothache and skin infections.

Previous phytochemical studies on the aerial parts of *A. emirnense* led to the isolation of nineteen (19) compounds, primarily including flavonoids, lignans, coumarins, anthraquinones, steroids, triterpenes, and iridoids [2]. However, to the best of our knowledge, no pharmacological or chemical investigations have been conducted on the roots of this species.

In this study, we report the phytochemical investigation, isolation, and structural elucidation of five secondary metabolites: 6-hydroxycoumarin (3), 6-hydroxyrubiadin (1), dehydro-α-lapachone (2), 8-acetyl-1-epishanzigenin methyl ester (4), and 8-acetylshanzigenin methyl ester (5), isolated from the ethyl acetate and methanol extracts of the roots of *A. emirnense*.

The aim of this research was to validate the traditional medicinal use of *A. emirnense* roots by determining their total phenolic content, evaluating their antioxidant activity, and isolating bioactive secondary metabolites from their ethyl acetate and methanol extracts.

2. Materials and method

2.1. Materials

2.1.1. Plant material

The roots of *Anthospermum emirnense* Baker were collected in Tsaramandimby Ramainandro, Faratsiho District, Vakinankaratra Region, Madagascar, in April 2023. The plant specimen was identified by a botanist at the Botanical and Zoological Park of Tsimbazaza (PBZT). The roots were dried, ground into a fine powder, and stored in an airtight container for future use.

2.1.2. Animals and management

Adult female SWISS mice, weighing between 24 and 33 g, were used in this study. The animals were housed in standard polypropylene cages under a 12-hour light/dark cycle and provided with a standard diet (LFL 14/20) ad libitum, with free access to water. Acclimatization training sessions were conducted once per week, each lasting 5 minutes, in the experimental animal room. Mice were fasted for 18 hours prior to the final experiment.

2.1.3. General experimental procedures

All organic solvents were distilled prior to use. Extracts were fractionated by liquid chromatography on silica gel (Merck MN Silica Gel 60 M; particle size: 0.04–0.063 mm), eluted under atmospheric pressure. NMR data, including ^{1}H, ^{13}C, DEPT, $^{1}H–^{1}H$ COSY, $^{1}H–^{13}C$ HSQC, and $^{1}H–^{13}C$ HMBC, were recorded in deuterated solvents (CD_3OD, $CDCl_3$, or DMSO) using a Bruker 600 MHz NMR spectrometer (operating at 600.19 MHz for ^{1}H and 125.78 MHz for ^{13}C). Chemical shifts (δ) are reported in parts per million (ppm), using tetramethylsilane (TMS) as the internal standard. The structures of isolated compounds were confirmed by comparison of their spectroscopic data with literature values. Total phenolic content was determined using a Jenway 7200 UV-visible spectrophotometer. Thin-layer chromatography (TLC) was performed on aluminum-backed silica gel plates (Macherey-Nagel, SIL G/UV254, 0.20 mm). Spots were visualized under UV light at 254 and 366 nm, or by heating after spraying with a 20% (v/v) sulfuric acid solution.

2.1.4. Phytochemical screening

A hydroalcoholic extract (2.17%) was prepared from the dried and powdered roots. Phytochemical screening was performed as previously described, using specific reagents to qualitatively assess the presence of various compounds

[3]. These included alkaloids, iridoids, steroids, triterpenoids, saponins, polysaccharides, and polyphenols such as coumarins, flavonoids, leucoanthocyanins, and tannins.

2.1.5. Extraction, fractionation and isolation

The dried and powdered roots (209 g) were macerated successively in solvents of increasing polarity: hexane (0.75 L for 7 days), ethyl acetate (0.8 L for 7 days), and methanol (0.9 L for 6 days). The resulting solutions were filtered and concentrated under reduced pressure to yield three extracts: hexane (0.62%), ethyl acetate (1.35%), and methanol (0.56%).

The ethyl acetate extract (3.021 g) was subjected to column chromatography on silica gel (60 g of silica gel 60, column dimensions 80 × 2 cm), using a gradient elution of cyclohexane and ethyl acetate, affording 385 fractions of 10 mL each. Fractions with similar TLC profiles were combined and subjected to purification by crystallization. This led to the isolation of three compounds: V_1 (12 mg, white powder), V_5 (10 mg, yellow powder), and V_2 (52 mg, yellow powder), respectively obtained from fractions 21–23 (eluted with cyclohexane/ethyl acetate 50:50), 32–42 (cyclohexane/ethyl acetate 40:60), and 188–192 (cyclohexane/ethyl acetate 10:90).

The methanolic extract (2 g) was also subjected to column chromatography on silica gel (60 g of silica gel 60, 80 × 2 cm), using a gradient of ethyl acetate and methanol as eluent, yielding 350 fractions of 10 mL each. One compound, V7 (10 mg, whitish powder), was isolated from fractions 320–321 (eluted with ethyl acetate/methanol 20:80). The structures of the isolated compounds were elucidated by spectroscopic analysis and by comparison of their NMR data with those reported in the literature.

2.1.6. Total phenolic compounds determination

The total phenolic content of *Anthospermum emirnense* extracts was determined using UV-Vis spectrophotometry with the Folin–Ciocalteu reagent [4]. A calibration curve was prepared using gallic acid standards dissolved in methanol at concentrations ranging from 0.00125 to 0.4 mg/mL. The plant extracts were prepared at a concentration of 0.5 mg/mL. For the assay, 200 µL of each sample or standard solution was mixed with 1.5 mL of 10% Folin–Ciocalteu reagent and 1.5 mL of sodium carbonate solution (0.06 g/mL). The mixtures were incubated in the dark for 1 hour at room temperature. Absorbance was then measured at 765 nm using a UV-Vis spectrophotometer, starting with the blank, followed by the least concentrated standard, and ending with the extract samples. The total phenolic content of the *A. emirnense* extract was expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/mg dry extract).

2.2. Biological tests

2.2.1. Antioxidant activity

Qualitative test

The free radical scavenging activity of the extract was evaluated using the bioautography method with DPPH [5]. Briefly, a DPPH solution in methanol (2 mg/mL) was prepared, and aluminium-backed silica gel plates were used. After application of the samples and development of the TLC plates, antioxidant compounds were visualized by spraying with the DPPH solution. The presence of antioxidant activity was indicated by yellow spots against a purple background.

Quantitative test

The quantification of the antioxidant activity was performed according to the methods of Brand-Williams et al. [6] and Sánchez-Moreno et al. [7], with some modifications. DPPH (25 mg) was dissolved in 100 mL of methanol, and the solution was stored in the dark. Ten milliliters of this stock solution were further diluted with 45 mL of methanol to prepare a 4.5% DPPH solution. Serial concentrations of the extract to be tested, ranging from 2 mg/mL to 0.125 mg/mL, were prepared.

In dry tubes, 200 µL of each extract concentration was mixed with 3800 µL of the 4.5% DPPH solution. Blanks containing 3800 µL of the 4.5% DPPH solution and 200 µL of methanol were also prepared. All tests were performed in triplicate and incubated in the dark for 60 minutes. The same procedure was applied to the control using ascorbic acid.

Absorbance was measured at 517 nm using a spectrophotometer. Antioxidant activity, expressed as the ability to scavenge free radicals, was estimated by the percentage of discoloration (percentage of inhibition) of the DPPH solution in methanol. The percentage inhibition was calculated using formula (1).

$$\text{Percent inhibition (\%)} = \frac{[(Ac - As)]}{(Ac)} \times 100 \quad \ldots\ldots\ldots \quad (1)$$

Ac = the absorbance of the control

As = the absorbance of the sample.

2.2.2. Acute toxicity study

The test was conducted according to the method described by Onifade et al. [8]. Mice were randomly assigned to control and treatment groups, with three animals per group. The ethanolic root extract was administered orally at doses of 250, 500, and 1000 mg/kg body weight. The control group received an equivalent volume of distilled water (10 ml/kg body weight). Following administration, the mice were observed continuously for 6 hours on the first day and then monitored daily for an additional 3 days.

3. Results

3.1. Preliminary phytochemical screening

Anthospermum emirnense Baker roots contained various constituents such as flavonoids, quinones; triterpenoids, saponins, polysaccharides, and other phenolic compounds.

3.2. Total phenolic compounds

The antioxidant capacity of plants is supported by phenolic compounds. Using the calibration curve for gallic acid, described by the equation y=0.257x+0.003 with an R^2 value of 0.996, the concentrations of phenolic compounds in crude ethanolic, ethyl acetate, and methanol extracts were determined. The total phenolic content (TPC) was calculated using the following formula:

$$CPC = \frac{mean\ concentration\ of\ sample}{mass\ of\ extract} \quad (2)$$

The phenolic compounds content have been determined as showing in table 1

Table 1 Content of phenolic compounds of the sample tested.

Sample	CPC (mg EAG/g sec extract)
Crude ethanolic	168
Ethyl acetate	34
Methanol	38

3.3. Biological tests

3.3.1. DPPH free radical scavenging activity

Qualitative test

Figure 1 Qualitative antioxidant test of crude ethanolic (EtOH), hexane, ethyl acetate (AcOEt) and methanol (MeOH) extracts of *Anthospermum emirnense* roots

Ethyl acetate (AcOEt), methanol (MeOH) and crude ethanol extracts (EtOH) of *Anthospermum emirnense* roots showed potency in scavenging of DPPH free radicals indicated by the yellow coloration (Figure 1).

3.3.2. Quantitave test

DPPH is a stable free radical that exhibits a maximum absorbance at 517 nm and is widely used to assess the free radical scavenging capacity or hydrogen-donating ability of compounds. It is a commonly employed method for evaluating the antioxidant activity of plant extracts and food substances [9]. The DPPH radical scavenging activity of *Anthospermum emirnense* root extracts (ethyl acetate and methanol) as well as the standard antioxidant, ascorbic acid, presents in Figure 2. It shows that the scavenging activity of *Anthospermum emirnense* root extracts against DPPH radicals exhibited a concentration-dependent response, quantified using the half-maximal inhibitory concentration (IC_{50}). This parameter denotes the antioxidant concentration required to reduce the initial DPPH concentration by 50%, where a lower IC_{50} value reflects superior antioxidant efficacy.

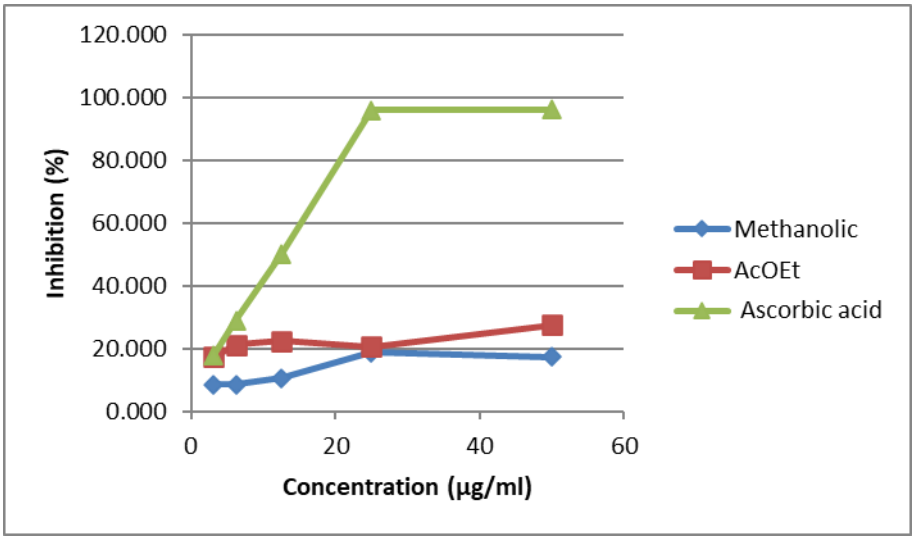

Figure 2 DPPH radical scavenging activity of extracts roots.

The table 2 summarizes the IC_{50} values for the tested extracts and reference compound. These results indicate that the ethyl acetate extract possesses stronger antioxidant activity than the methanol extract, though both were substantially less potent than ascorbic acid. This comparative analysis underscores the influence of solvent polarity on antioxidant extraction efficiency and highlights the potential of these *Anthospermum emirnense* root-derived extracts as moderate natural antioxidants.

Table 2 Inhibition concentration IC_{50} of the sample tested.

Sample	**CI_{50} (µg/ml)**
Ethyl acetate	155,02
Methanol	226,53
Ascorbic acid	12,18

In this study, the DPPH radical scavenging activity of the tested samples followed the order: ascorbic acid > ethyl acetate extract > methanol extract. According to this method, both the methanolic and ethyl acetate extracts of *Anthospermum emirnense* roots exhibited low antioxidant activity, with IC_{50} values greater than 100 µg/mL. The moderate antioxidant capacity observed in *A. emirnense* root extracts may be attributed to their low content of phenolic compounds.

3.4. Acute toxicity

No mortality occurred in any animal during the 72-hour observation period following single oral administration of *Anthospermum emirnense* ethanolic root extract at doses of 250, 500, and 1000 mg/kg, indicating a median lethal dose (LD_{50}) exceeding 1000 mg/kg.

However, dose-dependent behavioral alterations were observed. A reduction in locomotor activity was evident in mice receiving 500 and 1000 mg/kg doses. These effects manifested rapidly, with onset approximately 3 minutes post-administration, and persisted for 30 minutes. Complete behavioral normalization occurred in all animals thereafter, with no detectable residual effects.

3.5. Spectral data and identification of isolated compounds

The structures of the isolated compounds were determined through comprehensive analysis of their ^{1}H, ^{13}C, DEPT, HSQC, and HMBC NMR spectra, and by comparison with previously reported data in the literature. Compounds V_1, V_2, and V_5 were identified as dehydro-α-lapachone **(2)** [10]. 6-hydroxyrubiadin **(1)** [11,12,13] and 6-hydroxycoumarin **(3)** [14]. Respectively. Compound V_7 was found to consist of a mixture of iridoids, which appeared as a single spot on TLC when analyzed in various solvent systems, suggesting co-elution under those conditions. Compound V_7 is a mixture of 8-acetyl-1-epishanzigenin methyl ester **(4)** and 8-acetylshanzigenin methyl ester **(5)** [15,16,17]. The chemical structures of these compounds are presented in Figure 4.

6-hydroxyrubiadine (1): δ (ppm) ^{1}H NMR (600.19 MHz, CD_3OD): 8.05 (1H, d, H-8), 7.45 (1H, s, H-5), 7.15 (1H, s, H-4), 7.13(1H, d, H-7), 2.13 (3H, s, CH_3-2). δ (ppm) ^{13}C NMR (CD_3OD, 125.78 MHz): 186.1 (C-9), 182.3 (C-10), 162.8 (C-1), 162.2 (C-6), 162.8 (C-3), 135.1 (C-10a), 125.6 (C-4a), 128.2 (C-8), 126.6 (C-8a), 106.8 (C-7), 118.0 (C-2), 112.4 (C-5), 110.5 (C-9a), 107.3 (C-4), 7.3 (CH_3-2).

Dehydro-α-lapachone (2): δ (ppm) ^{1}H NMR (600.19 MHz, CD_3OD): 8.05 (1H, m, H-8), 8.03 (1H, m, H-5), 7.78 (1H, d, H-6), 7.77 (1H, d, H-7), 6.62(1H, d, H-1), 5.88 (1H, d, H-2), 1.50 (6H, s, 2CH_3-3). δ (ppm) ^{13}C NMR (CD_3OD, 125.78 MHz): 183.1 (C-9), 181.1 (C-10), 153.5 (C-4a), 135.3 (C-6), 134.3 (C-7), 132.9 (C-8a), 132.7 (C-10a), 132.2 (C-2), 127.1 (C-8), 126.8 (C-5), 118.8 (C-9a), 116.0 (C-1), 81.6 (C-3) , 28.1 2(CH_3-3).

6-hydroxycoumarine (3): δ (ppm) ^{1}H NMR (600.19 MHz, CD_3OD): 7.41 (1H, d, H-3), 6.96 (1H, s, H-5), 6.81 (1H, d, H-7), 6.73(1H, d, H-8), 6.12 (1H, d, H-4). δ (ppm) ^{13}C NMR (CD_3OD, 125.78 MHz): 165.7 (C-2), 143.3 (C-8a), 142.0 (C-4), 140.8 (C-6), 122.5 (C-4a), 118.1 (C-7), 111.3 (C-8), 110.7 (C-3), 109.8 (C-5).

8-acety-1-epishanzigemin methyl ester (4): δ (ppm) ^{1}H NMR (CD_3OD, 600 MHz): 7.29 (1H, s, H-3); 5.54(1H, d, H-1); 4.09 (1H, t, H-6); 3.63 (3H, s, H-11), 2.86 (1H, d, H-5); 2.69 (1H, dd, H-9); 2.11 (2H, s, H-7β); 1.83 (2H, d, H-7α); 1.93 (3H, m H-14); 1.40 (3H, s, H-12). δ (ppm) ^{13}C NMR (CD_3OD, 125.78 MHz): 170.0 (C-13); 168.3(C-10); 152.6 (C-3); 107.1 (C-4); 92.1 (C-1); 87.7 (C-8); 73.8(C-6); 50.0 (C-11); 49.1 (C-9); 46.5 (C-7); 41.1(C-5); 22.0 (C-14); 21.8 (C-12).

8-acetylshanzigemin methyl ester 5: δ (ppm) ^{1}H NMR (CD_3OD, 600 MHz): 7.44 (1H, s, H-3); 5.46 (1H, d, H-1); 4.16 (1H, q, H-6); 3,67 (3H, s, H-11); 2.84 (1H, t, H-5); 2.49 (1H, d, H-9); 2.11(1H, d, H-7β); 1.89 (1H, t, H-7α); 1.96 (3H, s, H-14); 1.56(3H, s, H-12). δ (ppm) ^{13}C NMR (CD_3OD, 125.78 MHz): 170.0 (C-13); 168.9 (C-10); 151.1 (C-3); 108.1(C-4); 90.0 (C-1); 89.7(C-8); 76.5 (C-6); 51.2 (C-11); 47.9 (C-9); 46.5 (C-7); 41.3 (C-5); 22.6 (C-12); 22.3 (C-14).

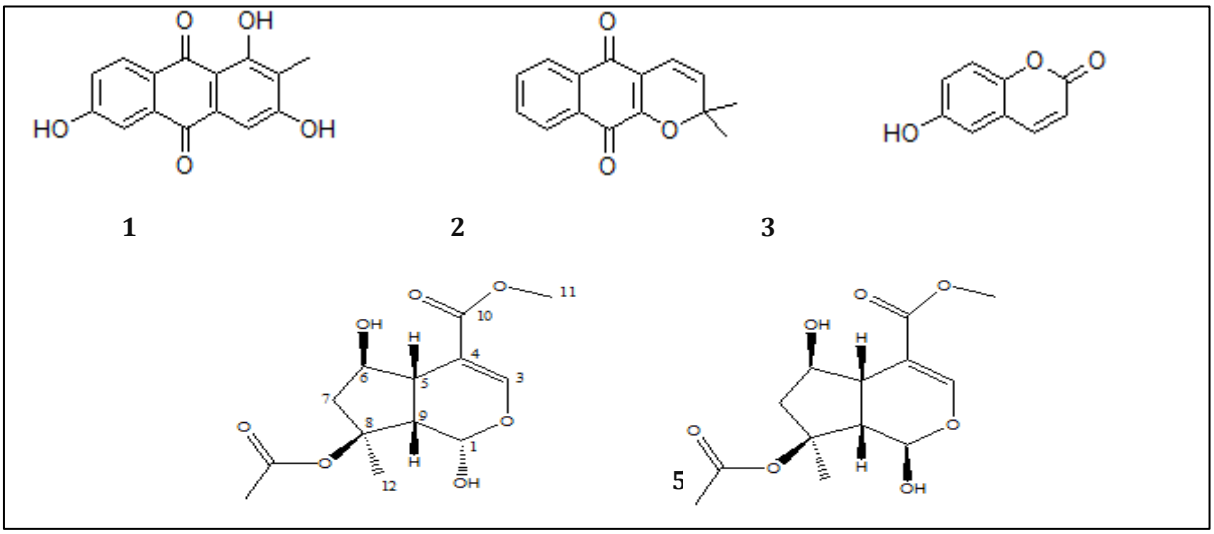

Figure 3 Structures of isolated compounds: 6-hydroxyrubiadine **(1)**, Dehydro-α-lapachone **(2)**, 6-hydroxycoumarine **(3)**, 8-acety-1-epishanzigemin methyl ester **(4)** and 8-acetylshanzigemin methyl ester **(5)**.

4. Discussion

This study on *Anthospermum emirnense* reports the isolation of five secondary metabolites from the roots: two iridoid isomers (4 and 5), one coumarin (3), one anthraquinone (1), and one naphthoquinone (2). The Rubiaceae family is well known for its biosynthesis of iridoid-type compounds, which supports the relevance of these findings. While previous investigations on the aerial parts of *A. emirnense* reported the presence of multiple iridoids [2]. This is the first report of such compounds from the roots of this species.

Among the isolated compounds, **6-hydroxyrubiadin (1)**, **6-hydroxycoumarin (3)**, **8-acetyl-1-epishanzigenin methyl ester (4)**, and **8-acetylshanzigenin methyl ester (5)** are reported here for the first time in the genus *Anthospermum*. **Dehydro-α-lapachone (2)**, on the other hand, has previously been reported in the roots of *Anthospermum pierreri* [18]. The isomeric iridoids (4 and 5) were earlier isolated as a mixture from *Morinda asteroscepa* [19], **6**-hydroxyrubiadin has also been isolated from *Rubia cordifolia***,** which belongs to the Rubiaceae family [20], but this the first report of the two product in the *Anthospermum* genus. The coumarin derivative 6-hydroxycoumarin (3) has been reported from *Prangos pabularia* (Apiaceae) [14], but this is its first report in the Rubiaceae family.

The concentration-dependent DPPH radical scavenging activity observed in *Anthospermum emirnense* root extracts aligns with established structure-activity relationships for phenolic antioxidants. The superior activity of the ethyl acetate extract (IC_{50} = 155.02 µg/mL) compared to the methanol extract (IC_{50} = 226.53 µg/mL) likely reflects differential solubility of antioxidant constituents. These disparities highlight the influence of phytochemical composition on antioxidant efficacy and underscore the need for compound-specific characterization. Also, non-polar solvents like ethyl acetate preferentially extract medium-polarity phenolics and flavonoids, which donate hydrogen atoms more efficiently to stabilize free radicals.

The absence of mortality at doses ≤1000 mg/kg in acute oral toxicity testing positions, *Anthospermum emirnense* ethanolic extract favorably against established safety thresholds. The LD_{50} exceeding 1000 mg/kg classifies it as "practically non-toxic" under the Hodge and Sterner scale (LD50 = 500–5000 mg/kg) [21]. The transient reduction in locomotor activity at 500–1000 mg/kg—onset at 3 minutes and resolution within 30 minutes—suggests reversible central nervous system (CNS) modulation rather than neurotoxicity.

The isolated compounds are known for various biological activities. **6-hydroxyrubiadin** has demonstrated anti-inflammatory activity [22], while **dehydro-α-lapachone** has been identified as a vascular-disrupting agent with potential anticancer properties [23]. These activities underline the pharmacological potential of *A. emirnense* root constituents and validate some of its traditional medicinal uses.

5. Conclusion

This study presents the first phytochemical investigation of the root extracts of *Anthospermum emirnense* Baker. The roots were found to contain a range of secondary metabolites including phenolic compounds, iridoids, anthraquinones, coumarins, and naphthoquinones. Although the total phenolic content was moderate, the extracts exhibited relatively low antioxidant activity, with IC_{50} values exceeding 100µg/mL, indicating limited free-radical scavenging potential.

Despite the modest antioxidant results, the isolation of known bioactive compounds such as 6-hydroxyrubiadin, dehydro-α-lapachone, and 6-hydroxycoumarin supports the ethnomedicinal use of this plant. Importantly, four of the five isolated compounds are reported here for the first time from the genus *Anthospermum*, highlighting the novelty of this research. The results provide a scientific basis for the traditional use of *A. emirnense* roots in Malagasy medicine and indicate its potential value in future phytopharmaceutical research.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declared no conflict of interest.

Statement of ethical approval

This study obtained approval from the Scientific and Ethical Review Committee of the Faculty of sciences, University of Antananarivo - Madagascar. The animals were treated with care throughout the study period and were kept in a well-controlled area according to the National Guidelines for the Care and Use of Laboratory Animals.

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